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This *Journal* is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The *Journal* publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this *Journal*.

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

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Editorial Views and News

As must be apparent from this first issue of 1960, several minor changes have been made in the general appearance of the *Journal.* Beginning with Volume **39,** the *Journal of Dental Research* is now being produced in conjunction with the University of Chicago Press. Certain elements of style are in conformity with the thirty other scholarly periodicals processed by this academic press in Chicago. It might be well to mention that the University of Chicago Press is a non-profit, education publication institution of considerable significance. There are over 78,000 scholars, libraries, and other institutions throughout the world who subscribe to University of Chicago Press journals. This Press has its own printing plant, where the *Journal of Dental Research* is being printed along with scores of books and other journals, covering an exceedingly wide range of subjects in the sciences and humanities. Warehousing is available for storing overruns and back numbers of the *Journal.* The Educational Directory, affiliated with the Press through the Association of American University Presses, has the names of some 200,000 scholars on mailing stencils. This is available for mail promotion, together with special mailing lists covering a great variety of professional categories. For the University of Chicago Press, the Cambridge University Press acts as agent in obtaining journal subscriptions from most parts of the British Commonwealth, while the McGraw-Hill Export Company of New York, the Maruzen Company of Tokyo, and hundreds of smaller agencies fill this function in other overseas regions.

In the same area of thought, it must be mentioned that a considerable amount of gratitude should go to the C. V. Mosby Company for its commendable work in publishing the *Journal of Dental Research* for the Association for the last 12 years. During this period there were issued some 72 numbers containing 1,271 manuscripts and 11,311 pages. This was a notable period in the expansion of dental research reportorial activity. Reasons for the change in publishing the *Journal* have been discussed intensively and extensively. There are two reasons, primarily. With its close physical proximity to the editorial office of the *Journal* at the Zoller Dental Clinic (just across the street), a much closer liaison can be established with the University of Chicago Press. This is resulting in greater expediency in producing the *Journal.* Approaching its fortieth year and having attained a considerable degree of maturity, the International Association of Dental Research, it was felt, could take the full share of responsibility for its official publication. Several other scientific societies have taken similar steps. Under the new arrangement, the I.A.D.R. becomes the publisher and the University of Chicago Press the printer and distributing agent. This entails greater responsibility in both an editorial and a financial sense, but, with all the friends the *Journal* has acquired, this greater responsibility poses no forseeable problems. As before, the Association is also the copyright holder and owner of the *Journal.* As a third and minor reason for the change, it is hoped that a university press, already concerned with some thirty other journals, can motivate the production of a more scholarly publication with emphasis on basic research concerned with the oral area.

On a historical note, the *Journal,* previous to 1948, was printed by the Waverly Press, the Williams and Wilkins Company, of Baltimore. From its founding in 1919 through the year 1947, there were only 26 volumes of the *Journal* with 15,048 pages containing research reports of various kinds.

Grants to the Journal for 1960.—A line of thanks should go to the National Advisory Dental Research Council, U.S. Public Health Service, for its grant of $4,000.00 toward publishing additional research reports, particularly of the rapidly printed, short-note variety. Special thanks should go to the William J. Gies Foundation for its grant of $1,500.00 toward the purchase of back issues of the *Journal* from the previous publisher. A number of Supporting Associates again have contributed funds in multiples of $100.00, including some noncommercial groups now being designated as Academic Supporting Associates of the *Journal.*

—F. J. O.

ANNOUNCEMENT OF 1960 GENERAL MEETING INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

The 38th General Meeting of the International Association for Dental Resarch will convene at the Morrison Hotel, Chicago, Illinois, on Thursday, Friday, Saturday, and Sunday, March 17, 18, 19, and 20, 1960. The Dental Materials Group of the Association will meet on Friday, Saturday, and Sunday, March 18, 19, and 20, 1960. The American Association of Dental Schools will meet at the same place beginning Monday, March 21, 1960.

Under the new arrangements of the *Journal* subscriptions, members must pay their dues early in the year in order to continue receiving the *Journal*.

DAN Y. BURRILL
Secretary-Treasurer

Instructions to Authors

1. *Correspondence.*—All manuscripts and correspondence regarding manuscripts should be addressed to the Editor: Dr. F. J. Orland, Zoller Memorial Dental Clinic, Univeristy of Chicago, 950 East 59th Street, Chicago 37, Illinois.

2. *General policy.*—All manuscripts submitted to the *Journal of Dental Research* will be acknowledged and, upon proper review, will be (*a*) accepted, (*b*) returned for revision, or (*c*) rejected.

Since copyright of the *Journal* is held by the International Association for Dental Research, the Editor must grant permission for republication or reproduction of all material originally appearing in the *Journal.*

3. *Manuscripts.*—Manuscripts submitted for publication should be typewritten on $8\frac{1}{2} \times 11$-inch paper, good bond paper, not slick or flimsy, double-spaced for footnotes and references as well as text, with 1-inch margins. The original typewritten copy and a carbon copy of the manuscript, together with two copies of all illustrations, should be submitted. The author should always retain a third copy. Manuscripts and illustrations cannot be returned until after the article is published, and then only if requested.

Following a concise introduction, a manuscript should, where possible, be divided into the following parts: Materials and Methods, or Experimental Methods; Results; Discussion; Summary; References.

The title of the paper should be followed by the authors' names and professional addresses. Academic degrees will not be published because these can usually be ascertained from a biographical directory. Change of authors' address since writing should be placed in a footnote. Reference to a research grant should include its number and must be cited in a footnote. For example: "This study was supported by Research Grant D-222 from the N.I.H., U. S. Public Health Service."

In addition, a running head or short title of not more than five words should be provided.

The Editor reserves the right to make minimal literary corrections.

4. *Illustrations, Figures, and Tables.*—A limited number of halftone (black and white) and zinc engravings are reproduced free to the author. The cost of illustrations (especially color reproductions) requiring more involved and expensive processes must be borne by the author or his institution.

All illustrations and tabular matter must be in finished form for the printer, and not larger than $8\frac{1}{2} \times 11$ inches. Original photographs and artists' drawings should be submitted if possible. When they are not available, good photographic copies on glossy paper can be reproduced. Line drawings and graphs should be executed on white paper in black India ink with the sharpest possible lettering.

Each illustration should be numbered lightly on the back and should have a suitable legend referring it to the text. Legends for illustrations should be listed on a separate sheet, and should be understandable without reference to the text. Tables should be typed on separate pages, one table to a page. Figures and tables are referred to with Arabic numerals.

5. *References.*—References should be listed at the end of the article in the order in which they appear in the text and with a number corresponding to a superscript in the text. References to periodicals should take the following form: Name of author(s) with initials following surname, full title of paper, name of journal in accepted abbreviated form, volume number, first page of paper, and year. References to books should include the author's name, title of the book, page, city of publication, publisher, and year of publication. Examples:

1. To a periodical: HECK, T. O. Dental Disease, *J. D. Res.*, **33**:889, 1912.
2. To a book: WARE, B. E. *Oral Problems,* p. 114. Chicago: Y. B. Good & Sons, 1950.

The accepted abbreviated form for dental journals is that used in the *Index to Dental Literature,* compiled by the American Dental Association. For other journals, the usage in the *List of Periodicals Abstracted by Chemical Abstracts* should be followed. The author is responsible for the accuracy of his references. Authors should consult current issues of the *Journal* for further details covering the accepted form.

Works cited in the text by reference to their authors should in the first instance be referred to with the names of all authors. Subsequent mention may be abbreviated to "*et al.*"

References cited should be restricted to closely pertinent papers. Articles cited in the references should be either "accepted for publication" in a specific journal or already printed.

6. *Reprints.*—A limited number of reprints of articles can be purchased by authors through the Editor. A table showing cost of reprints and an order slip will be sent with galley proofs.

7. *Abbreviations and Technical Terms.*—The use of the following abbreviations in the text of the manuscript will minimize editorial corrections. When referring to actual measurements, cm. = centimeter(s), $cm.^2$ = square centimeter(s), gm = gram(s), kv. = kilovolt(s), mg. = milligram(s), μg = microgram(s), ml. = milliliter(s), mm. = millimeter(s), μ = micron(s), mμ = millimicron(s), mEq. = milliequivalent, N = normality, M = molar, ppm. = parts per million, rpm = revolutions per minute, psi = pounds per square inch, 37° C. = 37 degrees centigrade. Numbers should be in Arabic numerals. The following words should be spelled out in the text but may be abbreviated or replaced by symbols in tables: per cent, number, minutes, seconds, concentration.

Proprietary substances, trade names, and sources of supply should appear in footnotes. Where possible and appropriate, substances should be referred to in the text by their generic or full chemical names.

Microörganisms should be referred to in accordance with the International Rules of Nomenclature. When applicable, the nomenclature for bacteria presented in *Bergey's Manual of Determinative Bacteriology* (7th ed., 1957), will be followed.

8. *Research Annotations.*—Manuscripts submitted as short research notes must be not more than 500 words in length. If illustrative material is submitted, the text should be correspondingly shorter because only one page is allowed to each article. These notes should present results of original work together with adequate data. Contributors should follow the format and bibliographical form used for Research Annotations in current issues of the *Journal.*—F. J. O.

The Incidence of Caries in Sialoadenectomized Rats Drinking Water Containing Human Saliva

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Although the physical properties, chemical nature, and microbial composition of caries-immune and caries-susceptible saliva has been the subject of extensive investigation, relatively little work has been done on the effects of the addition of saliva to the dietary regimen on the carious process in the experimental animal. Granados, Glavind, and Dam[1] studied the effects on the dental caries activity in hamsters of adding unstimulated and untreated saliva from two human subjects who had markedly different caries susceptibilities. The animals receiving the saliva from the caries-resistant subject exhibited less caries. It was concluded that the saliva from the caries-resistant individual contained a factor (or factors) which was able to decrease dental caries activity.

It appeared important to confirm, on an in vivo basis, that a difference existed between the salivas of caries-immune and caries-susceptible individuals. In this paper the effect of the addition of saliva from caries-immune and caries-susceptible individuals on the dental caries experience in sialoadenectomized albino rats is reported.

EXPERIMENTAL METHODS

One hundred and twenty weanling rats† were divided into six experimental groups, each group consisting of an equal number of males and females. Three groups of these animals were sialoadenectomized, one group was sham-operated, and the remaining two served as the intact control groups. The surgically operated groups were allowed a period of 10–12 days for recovery before being placed on their dietary regimens.

Stimulated whole saliva was collected on a daily basis, five times per week, from two groups of human donors. The caries-immune group consisted of 25 donors, six to twenty-one years of age, who showed no evidence of carious lesions (restored or unrestored) by clinical and radiographic examination. The caries-susceptible group was made up of approximately 80 donors, Freshman dental students, with histories and evidence of recent caries experience. The saliva was collected from the individuals in each group by paraffin stimulation for 15–20 minutes, pooled by group, passed through gauze to remove gross particulate matter, and stored under refrigeration at 4° C. for a maximum of 6 hours. An aliquot of each of the pooled salivas was diluted with an equivalent amount of tap water daily and fed to the appropriate experimental group.

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† Sprague-Dawley strain, furnished by Blue Spruce Farms, Altamount, New York.

A modified Stewart, Hoppert, and Hunt,[2] powdered, purified ration consisting of confectioner's sugar, 67 per cent; whole powdered milk, 20 per cent; desiccated liver, 3 per cent; alfalfa, 5 per cent; brewer's yeast, 2 per cent, and salt mixture,* 3 per cent, was utilized. This diet will henceforth be referred to as "Ration 19" (Table 1).

All animals were housed in individual cages with raised screen bottoms and received their respective diets and drinking fluid ad libitum for a period of 12 weeks. At the end of the experimental period, the animals were sacrificed by ether inhalation. The extent of dental caries was scored by the method described elsewhere.[3]

TABLE 1

EXPERIMENTAL DESIGN AND CARIES INCIDENCE

Experimental Group	No. of Animals*	Condition of Animal	Diet	Fluid ad Libitum	ACM†	ACL‡	AECL§
I.....	20	Normal	Ground Purina chow	Tap water	1.85	1.85	2.40
II.....	18	Normal	Ration 19	Tap water	9.55	11.28	17.44
III.....	14	Sialoadenectomized	Ration 19	Tap water	11.50	13.33	55.92
IV.....	14	Sialoadenectomized	Ration 19	Tap water + "susceptible" saliva, 1:1	11.93	23.57	109.57
V.....	16	Sialoadenectomized	Ration 19	Tap water "immune" saliva, 1:1	10.88	15.69	37.50
VI.....	16	Sham-operated	Ration 19	Tap water	11.06	12.56	17.87

* Surviving at the end of the experimental period.
† Average number of carious molars.
‡ Average number of carious lesions.
§ Average extent of carious lesions.

RESULTS

A summary of the dental caries experience in each of the experimental groups is presented in Table 1, and statistical treatment of these data is given in Table 2.

The cariogenic diet, Ration 19, produced significantly greater incidence of dental caries in the normal and sham-operated groups than was evident in the controls, which were fed ground Purina chow. Desalivation resulted in a significantly greater extent of carious lesions. The addition of saliva from caries-susceptible individuals to the drinking water fed to the desalivated animals resulted in a still greater number of carious teeth, number of carious lesions, and relative amounts of tooth structure involved in the destructive process (Table 1). The statistical methods were applied only to the extent of carious lesions in the various groups (Table 2). Although there were no significant differences between the extent of caries in the desalivated animals of Group III, which received only tap water, and those of Group V, which received caries-immune saliva, a trend toward lower caries incidence was apparent in the latter group. However, a comparison of the caries-susceptible saliva group (Group IV) with the caries-immune saliva group (Group V) showed that a highly significant difference exists between them. Sham operation apparently did not influence the carious process beyond that which was seen in the intact experimental control on the cariogenic diet.

Figure 1 presents in graphic form the average total, mandibular, and maxillary caries scores for each experimental group.

* Hubbell, Mendel, and Wakeman (H.M.W.) salt mixture available from Nutritional Biochemicals Corp., Cleveland 28, Ohio.

TABLE 2

STATISTICAL COMPARISON OF CARIES EXPERIENCE IN VARIOUS GROUPS

Animal Group	No. of Animals	Average Extent of Carious Lesions	S.E.M.*	*t*-Ratio	*P*-Value
I.....	20	2.40	0.64		
				10.81	$P \ll 0.001$
II.....	18	17.44	1.23		
				2.87	$0.01 > P \gg 0.001$
III.....	14	55.92	13.38		
				2.64	$0.05 > P > 0.01$
IV.....	14	109.57	15.58		
				4.28	$P \ll 0.001$
V.....	16	37.15	6.63		
				1.27	$0.3 > P > 0.2$ not significant
VI.....	16	17.87	2.15		
				6.84	$P \ll 0.001$

* S.E.M. = Standard error of the mean.

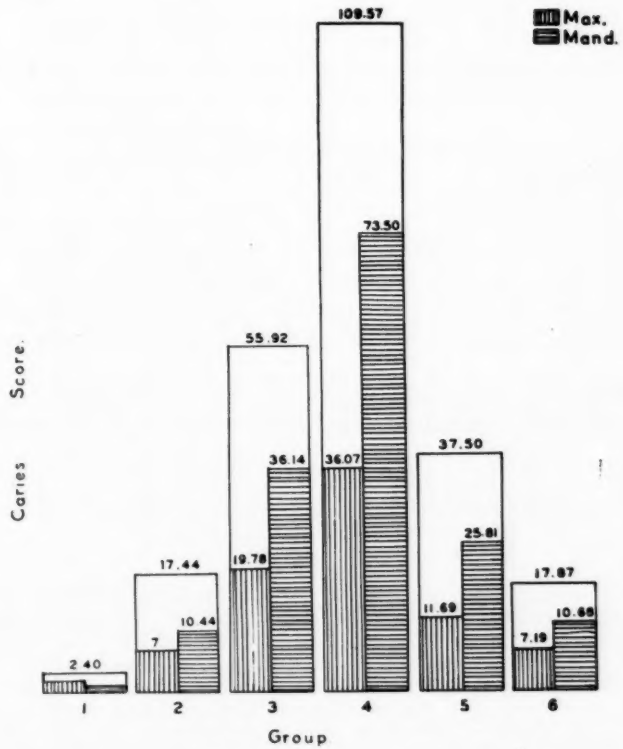

FIG. 1.—Comparison of histograms of the average total, mandibular and maxillary caries scores in each experimental group.

The distribution and extent of carious tooth involvement in each experimental group according to sex is given in Table 3. Although the female animals of Group III appeared to have a greater caries incidence than the males, this difference was not statistically significant.

The effect produced by the addition of caries-susceptible saliva to the drinking water of the sialoadenectomized rats of Group IV is of considerable interest. The donors of this saliva were male dental students, and the greatest increase in caries incidence—over threefold—occurred in the sialoadenectomized male rats on this saliva-water mixture as compared to the sialoadenectomized Group III male animals on water alone. This increase was found to be significant at the 5 per cent level.

It may be noted in Table 4 that the mandibular first and second molars showed the highest degree of susceptibility and extent of caries, followed in sequence by the maxil-

TABLE 3

CARIES SEVERITY SCORES IN EACH GROUP RELATIVE TO SEX DIFFERENCES

	GROUP					
	I	II	III	IV	V	VI
Males	3.5	14.6	32.8	106.6	37.2	18.4
Females	1.3	21.0	68.8	111.2	37.7	17.4

TABLE 4

DISTRIBUTION OF OCCLUSAL CARIES

MOLARS	GROUP											
	I		II		III		IV		V		VI	
	R	L	R	L	R	L	R	L	R	L	R	L
Maxillary:												
M_1	0.00	0.00	0.61	0.67	4.07	2.50	6.21	5.43	1.63	1.63	1.00	0.81
M_2	0.05	0.10	0.94	0.89	4.14	2.57	6.21	6.21	2.19	2.06	1.06	1.06
M_3	0.50	0.85	2.00	1.89	3.43	3.07	5.36	6.57	2.00	2.00	1.63	1.63
Mandibular:												
M_1	0.30	0.40	2.56	2.72	8.71	7.85	18.00	17.71	6.25	5.00	2.06	2.06
M_2	0.10	0.10	2.22	2.06	7.21	7.43	14.86	14.57	5.56	5.63	2.19	2.75
M_3	0.00	0.00	0.38	0.50	2.36	2.57	4.64	4.36	1.94	1.63	0.81	0.85

TABLE 5

BILATERAL OCCURRENCE OF CARIOUS LESIONS

	MAXILLARY			MANDIBULAR		
	M_1	M_2	M_3	M_4	M_5	M_6
Right	2.01	2.19	2.71	5.70	4.86	1.51
Left	1.65	1.95	2.50	5.38	4.80	1.48

lary third, second, and first molars and the mandibular third molar. Moreover, the extent of carious involvement in the mandibular first molar of the sialoadenectomized animals was two to three times greater than that in the maxillary first molar.

The mean caries scores of the total data are given for each molar tooth in each arch in Table 5. A bilateral symmetry in the occurrence of dental caries is evident, with a trend toward increased caries incidence in the right maxillary and mandibular molars.

DISCUSSION

It appears from the data presented that the addition of human saliva to the drinking water fed to the sialoadenectomized rats maintained on a cariogenic diet modified their dental caries experience.

Since the fluid intake of all three groups of sialoadenectomized animals was comparable and since the salivary flow, while very slight, was presumably constant, it is concluded that the extension of the carious lesions was modified by a factor or factors in the fed saliva per se. These salivary factors may include buffering capacity,[4] action of the oral microbiota,[5, 6, 7] physical properties,[8] hormonal content,[9] enzymes,[10] and effects on enamel maturation.[11] The sialoadenectomized animal may provide a means for studying these factors.

SUMMARY

Sialoadenectomized Sprague-Dawley albino rats maintained on a cariogenic diet showed a statistically significant increase in the incidence of dental caries as compared to intact and sham-operated controls.

The addition of pooled saliva from caries-susceptible individuals to the drinking water fed desalivated rats resulted in a significant increase in tooth destruction. Pooled saliva from caries-immune individuals resulted in a decrease in the average extent of carious lesions that was not statistically significant. Real differences in caries incidence were noted between the sialoadenectomized animals receiving caries-immune saliva and those receiving caries-susceptible saliva. It was concluded that saliva contains a factor or factors which modify the extension of dental caries in the sialoadenectomized albino rat.

A high degree of bilateral symmetry and sexual similarity in the distribution of carious lesions was found. Data on the distribution of occlusal caries in the molar teeth are presented.

The assistance of Elena Bungener, D.D.S., in this study is gratefully acknowledged.

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Fluoride Uptake by Enamel from Certain Dental Materials

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Previous research[1] from this laboratory was concerned with the effects of various dental materials on the solubility of tooth tissue. Many of the materials tested altered the solubility of both powdered and intact enamel and dentin. Solubility was reduced appreciably after contact with some of the materials, and this reduction appeared to be associated with the presence of fluoride.

In view of these observations, the research was continued to determine something of the basic mechanism involved and to establish more definitely the role of fluoride in this phenomenon. The specific purpose of this study was to measure the fluoride leached from the materials to the surrounding media and the subsequent change in the fluoride content of the exposed enamel.

MATERIALS AND METHODS, PROCEDURE

The procedure was basically the same as that employed in the previous research.[1] In studying the release of fluoride, 20-mm. disks of the material were utilized. One hour after preparation these disks were suspended by stainless-steel wires in crucibles containing 15 ml. of glass-distilled water. The disks were immersed in the water for a period of 5 days, the solution being changed at 24-hour intervals. This test period was adopted because preliminary data had not indicated appreciable dissolution after this time. Each day the media were collected and quantitatively analyzed for the presence of fluoride. Thus it was possible to obtain information concerning the rate of solution of the fluoride, as well as the total amount released.

Where the uptake of fluoride by powdered enamel was investigated, disks of the material were stored in crucibles containing 15 ml. of water and 130 mg. of powdered enamel. The water was again changed daily during the 5-day period. At that time the enamel was washed, dried, weighed, and the fluorine content determined.

For comparative purposes one group of six specimens was treated for 20 minutes with an unbuffered 2 per cent aqueous solution of sodium fluoride.

All the enamel samples were taken from a common pool. Fifty untreated specimens, serving as controls, were analyzed for fluoride. The powdered enamel was prepared by

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crushing dried, extracted teeth. The particle size used was that which would pass a 100-mesh screen and be retained by a 140-mesh. Enamel was separated from the other components by a combination of two methods. The separating medium was that described by Manley and Hodge,[2] whereas a separatory funnel was utilized, as suggested by Battistone and Burnett.[3]

The analytical procedure was one involving the microdistillation of the samples as outlined by Singer *et al.*[4] Fluoride was measured by a modification of the spectrophotometric procedure of Megregian,[5] utilizing a zirconium-eriochrome cyanine R lake. This modification consisted of a change in dilution of the products of distillation, here necessitated because of the substitution of a Model DU spectrophotometer for a Model B.

TABLE 1

Materials	Company
S. S. White filling porcelain	S. S. White Dental Mfg. Co.
Ames Berylite	W. V. B. Ames Co.
FluorOn	American Consolidated Dental Co.
Resin+5% NaF*	L. D. Caulk Co.
Resin+2% NaF*	L. D. Caulk Co.
Zinc phosphate cement	S. S. White Dental Mfg. Co.
Zinc phosphate+10% CaF_2*	L. D. Caulk Co.
Kryptex	S. S. White Dental Mfg. Co.
2% NaF	J. T. Baker Chemical Co.

* Per cent fluoride added to powder.

TABLE 2

Material	Av. Wt. of Disks* (Gm.)	Dissolution of Fluorine with Time (ppm/ml) Days					Total
		1	2	3	4	5	
Silicate	0.7	56.0	42.4	31.4	22.9	14.1	166.8
FluorOn	0.9	32.8	8.9	7.1	5.8	5.7	60.3
Kryptex	1.0	28.3	15.3	9.3	7.4	9.2	69.5
Resin+5% NaF	0.9	8.3	0.7	0.5	0.8	0.6	10.9
Resin+2% NaF	0.9	1.1	0.1	0.1	0.1	0.8	2.2
Zinc phosphate+10% CaF_2	1.1	0.3	0.3	0.3	0.5	0.4	1.8
Ames Berylite	0.8	0.0	0.0	0.0	0.0	0.0	0.0
Zinc phosphate	1.1	0.0	0.0	0.0	0.0	0.0	0.0
Distilled water	.....	0.0	0.0	0.0	0.0	0.0	0.0

* Minimum of eight samples.

At least four specimens were used for each material tested. The various materials included in this investigation are shown in Table 1. The handling and manipulation were carefully standardized, and specific powder-liquid ratios were employed for each material. It was important that the amount of powder used in preparation of the disks be accurately controlled because the powders contained the fluorides. It was thus possible to prepare disks of a given material to contain the same quantity of fluoride. It was equally important that the size of the specimens be carefully standardized, since research has shown that surface area exerts a pronounced effect on solubility measurements.[6]

RESULTS

Fluorine release by materials.—The mean values showing the release of fluorine from the various materials can be seen in Table 2. It is app ent that the greatest dissolu-

tion of fluoride occurred during the first 24 hours. Generally, the amounts released diminished with time. Silicate specimens liberated fluoride in relatively large amounts throughout the test period, although the trend of decreasing dissolution was again noted.

Silicates gave off the largest amounts of fluorine, approximately three times the amount lost by Kryptex and FluorOn. The fluorine released by the experimental resins was relatively small in comparison. Although the zinc phosphate cement containing 10 per cent CaF_2 lost almost the same total amount over the 5-day period as did the 2 per cent NaF resin, the pattern of dissolution was quite different. Half the total fluoride released was dissolved from the 2 per cent NaF resin during the first 24 hours, whereas the zinc phosphate containing 10 per cent CaF_2 lost fluoride uniformly but in very small amounts throughout the entire test period. No fluoride was detected in media in which the fluoride-free zinc phosphate cement and Ames Berylite were stored. Control tests on distilled water used for storage revealed no measurable fluorine.

The amount of fluorine contained in the disks prepared from each material was calculated on the basis of the percentage fluorine present in the material* and the speci-

TABLE 3

Materials	Av. ppm
Untreated	120
S. S. White silicate	712
FluorOn	626
Kryptex	248
5% NaF resin	186
2% NaF resin	171
Zinc phosphate + 10% CaF_2	97
Ames Berylite	88
Zinc phosphate	77
2% NaF	248
Distilled water	120

men weight. These estimations indicate that a very small percentage of the total fluoride content of the specimens was released to the surrounding medium. Only about 0.15 per cent of the total amount present in silicate cement was leached out. Approximately 1 per cent was dissolved from the 2 per cent NaF resin, while 2 per cent was released by the 5 per cent NaF resin. Kryptex gave up 0.02 per cent fluorine, and the 10 per cent CaF_2 zinc phosphate cement liberated 0.002 per cent. Owing to the unhomogenous distribution of fluoride in the FluorOn powder, no attempt was made to obtain information on this material. It should be emphasized that these figures on percentage release of fluoride are only approximations but are included in order to illustrate the minuteness of the quantity of fluorine that becomes available to the surrounding medium or tooth tissue.

Uptake of fluoride by powdered enamel.—The untreated enamel, which served as the control, was found to contain an average of 120 ppm fluoride. This figure compares with that of previously reported data.[7–11] The fluoride content of powdered enamel after treatment with the various dental materials can be seen in Table 3. As a further control, eight enamel samples were placed in distilled water. These samples showed no measurable change in fluoride content when stored in that medium.

* Percentages were based on the approximate amounts of fluoride salts employed by the manufacturer in the preparation of the materials.

The largest amounts of fluoride uptake were observed in enamel samples exposed to silicate and FluorOn. Storage with these materials resulted in fluoride increases of 495 and 423 per cent, respectively. Enamel subjected to Kryptex produced an increase of 208 per cent. There was a 43 per cent gain in enamel exposed to 2 per cent NaF resin, while a 54 per cent increase was induced by the 5 per cent NaF resin. These data are compared to the 207 per cent fluoride increase in powdered enamel treated for 20 minutes with the 2 per cent sodium fluoride solution.

Enamel stored with Ames Berylite, the experimental CaF_2 zinc phosphate cement, and the fluoride-free zinc phosphate cement actually decreased in fluoride content. At the end of the storage period the outer layers of the cement disks were removed by scraping. These scrapings, as well as the remaining bulk of the specimens, were analyzed for fluoride. These analyses indicated that the materials themselves had gained the greater part of the fluoride lost from the enamel. The fluoride concentration was greatest on the outer surfaces of the disks of the fluoride-free zinc phosphate cement, but some also permeated deeper into the material.

TABLE 4

Material	Available Fluorine (ppm)	Change in Enamel (ppm)	Material	Available Fluorine (ppm)	Change in Enamel (ppm)
Silicate	3,300	593	Zinc phosphate+10% CaF_2	57	− 19
FluorOn	1,810	506	Ames Berylite		− 26
Kryptex	1,280	128	Zinc phosphate		− 35
Resin+5% NaF	120	66	2% NaF	1,400	128
Resin+2% NaF	66	51			

DISCUSSION

The data indicate that the fluorine content of enamel is altered by exposure to various dental materials. In Table 4 the quantity of fluoride dissolved from the materials is compared with the change induced in the powdered enamel. Generally, the materials releasing the most fluorine also produced the highest fluoride uptake in the enamel. However, it can be noted that wherever large quantities of fluoride were present in the solution, only a small percentage was actually utilized by the tooth tissue. For example, only 18 per cent of the available fluoride was found in enamel stored with silicate cement. Where smaller amounts were in solution, as with the fluoride resins, a much greater percentage was found in the enamel. This increase was approximately 80 per cent in the case of the 2 per cent NaF resin. These data are supported by previous investigations on the uptake of fluorine as influenced by solution concentration.[12-13] These studies have reported that the lower the concentration of fluorine, the greater the percentage uptake by powdered enamel. Other investigations on powdered enamel have shown that increases in the concentration of fluorides in solution tend to produce greater reduction in enamel solubility only up to certain levels.[1, 14] Beyond these levels increases in the amount of fluoride in the solution has no added effect on the solubility.

There are undoubtedly many factors which govern the availability of the fluorine to the enamel. Often the fluorides are present in the form of relatively stable compounds.

Furthermore, the products of the dissolution of some materials may well contain interfering ionic systems and thus reduce the availability of the fluorine. On the other hand, there may be ions which accentuate the effects of fluorine. This could explain some of the observed differences.

The data clearly indicate that fluoride in appreciable quantities is leached out of some dental materials and that this fluoride can react with powdered enamel. These data support the previous studies showing that the solubility of tooth structure is reduced through contact with certain materials as related to the fluoride content of enamel. A comparison of enamel solubility data taken from the previous publication and the data on the fluorine content of powdered enamel tend to support this statement (Table 5). In most instances, the greatest reduction in enamel solubility was associated with the materials which produced the largest increase in the fluoride content of powdered enamel.

TABLE 5

Material	Fluorine Content (ppm)	Reduction in Calcium Solubility (Per Cent)	Material	Fluorine Content (ppm)	Reduction in Calcium Solubility (Per Cent)
Silicate	712	34	Zinc phosphate+10% CaF_2	97	10
FluorOn	626	45	Ames Berylite	88	11
Kryptex	248	14*	Zinc phosphate	77	1
Resin+5% NaF	186	19	2% NaF	248	16
Resin+2% NaF	171	20			

* Intact enamel.

SUMMARY

The analysis of over 400 samples of powdered enamel and 2,500 spectrophotometric determinations led to the following conclusions:

1. Generally the release of fluoride from the materials occurred relatively fast, the amounts diminishing with time.

2. Certain materials increased the fluoride content in powdered enamel, while fluoride-free materials actually reduced the fluoride in the enamel.

3. The increases in the fluorine content of enamel on exposure to various materials correlated with the previously reported reduction in enamel solubility.

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An Approach to Quantitative Electromyography of the Masseter Muscle

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Electromyographic data[1–5] representing muscle activity during movements of the mandible (e.g., chewing) are the result of several variables operating simultaneously—i.e., continual variation in muscle length, bite dimension, and temporomandibular joint axis. Such data have contributed to our knowledge of the relative function of the masticatory muscles and have certainly indicated the potential value of electromyographic data in dentistry. However, the usefulness of electromyography depends upon the ability to characterize quantitatively the "normal" population. The data at the present time do not permit such a characterization, because of the lack of standardization in recording techniques and the obvious difficulty in expressing the resultant electromyograms in simple quantitative terms. Our studies were undertaken, with equipment that was already available, to determine the feasibility of an extended program which we felt could be justified only by reasonable assurance that standardization and quantification were practical.

MATERIALS AND METHODS

Surface electrodes were prepared by forming a circular disk 1 mm. thick and 3 mm. in diameter from a drop of solder. The leads from the electrodes were connected to a single-beam oscilloscope via an amplifier adjusted so that an input of 1 mV produced a 25-mm. deflection on the screen. A plastic bite block, 1 cm.^{2} and 6 mm. thick with electronic equipment to reflect pressures exerted on the block, was constructed in a manner similar to that of Howell and Manly.[6]

With the bite block placed between the most anterior teeth so that the upper and lower teeth opposed one another, the oral structures were fixed, and the bite opening remained constant regardless of the pressure exerted by the muscles. Two electrodes were attached to the left masseter muscle with adhesive tape, so that each electrode was located 1 cm. on each side of the masseteric nerve motor point (determined previously on the muscle) on an imaginary line drawn through the motor point parallel to the anterior margin of the ear. The third electrode—the ground—was placed in the center of the subject's forehead. Electrical contact was made with electrocardiograph

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paste. The subject, with his back to the scope, was asked to bite on the "block" until the pressure indicator reached a designated number and then to maintain the needle on that number. The electrical activity on the oscilloscope (the potential difference between the two electrodes on the masseter muscle) was recorded on film with a 16-mm. camera (16 frames/sec) for a period of 3 seconds, while the pressure was maintained by the subject. The subject relaxed after each pressure recording.

Since no base line is available on a single-beam scope during activity, a length of string was taped across the scope to coincide with the base line at rest. A 60-cycle record was taken to calibrate sweep time, and a separate record of 1-mV calibration was taken with the string base line as a reference point.

The camera speed and scope sweep were such that some film frames carried only fragments of the record. However, the record provided 6 frames or more with a full sweep for each pressure recorded. These frames were printed and enlarged. The areas above and below the base line were measured with a planimeter, averaged, and converted to millivolts, utilizing the calibration records made for each subject. The calibration records thus took into account variations in magnification due to placement of the camera and printing of the negatives, as well as changes made in the amplification sensitivity or sweep speed of the oscilloscope. Repeat records were taken at the same sitting and also at intervals of a month or more between sessions on some of the subjects, in an attempt to ascertain the variables of the technique.

Since we were interested, at this point, only in the procedure of handling electromyographic data, subjects were chosen only on the basis of their availability. Records were made and analyzed on a total of 40 subjects. After the data were quantitated, it was possible to recall 22 of the subjects to check their occlusion. The following observations were made on each subject: (1) the presence or lack of temporomandibular joint interference as indicated by an apparent irregular movement or noise associated with joint movement when the subject opened and closed the mouth; (2) the estimated amount of forward positioning of the mandible with the mouth closed (anterior displacement) in relation to the original arch of closure as determined by holding the mandible firmly and moving it up and down until apparently free from active muscle control; and (3) the type of occlusion classified according to alterations in the anteroposterior relationship of the lower arch to the upper arch.

RESULTS

Figure 1 illustrates a printed frame of the electrical activity as it appeared on the oscilloscope. To quantify this record, the following relationships were used:

k = Sweep distance (cm.) over which the planimeter area, S, was measured in square centimeters (Fig. 1);

t = Cm/cycle, determined from a 60-cycle record (Fig. 2)·

$60t$ = Cm/sec;

d = Deflection (cm.) for 1-mV input (Fig. 3)·

$60td$ = Area/mV × sec·

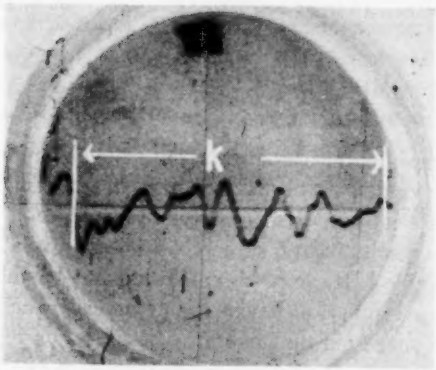

FIG. 1.—The electrical activity from the left masseter muscle with a force of 23 lb. exerted on the bite block.

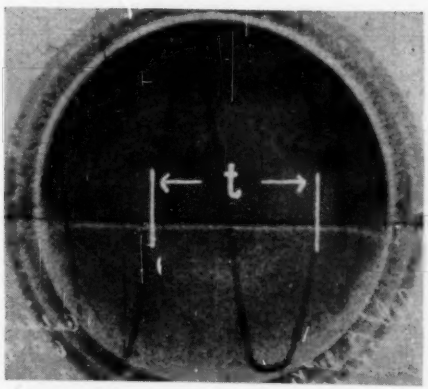

FIG. 2.—A 60-cycle/sec record on the oscilloscope

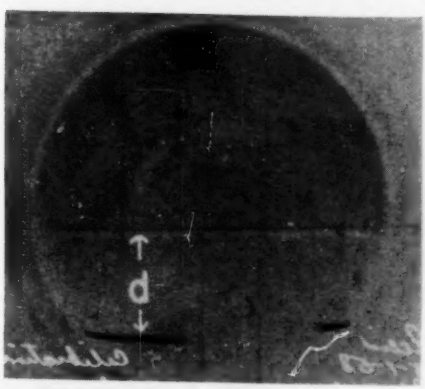

FIG. 3.—Deflection of the oscilloscope beam with an input of 1 mV

Thus,

$$\frac{60St}{k} \times \frac{1}{60td} = \frac{S}{kd} \quad \text{or} \quad \frac{\text{cm.}^2}{\text{cm.} \times \text{cm/mV}} = \text{mV}.$$

It was found that repeated measurements, once the electrodes were placed in position, produced similar values. However, considerable variation existed from one time to another—i.e., when electrodes were removed and replaced. Table 1 illustrates this variation. The average results from 6 subjects who were available for multiple recordings are presented in Table 2. It can be observed that the general nature of the response to various pressures was similar, although the numerical values differed from subject to subject. These data suggested the possibility of a relationship between our occlusal classifications and electrical activity. No more than 3 of the total subjects fell into

TABLE 1

ELECTRICAL ACTIVITY OF LEFT MASSETER MUSCLE AT VARIOUS ORAL FORCES EXERTED AT FIXED BITE OPENING OF 6 MM.

DATE*	FORCE (LB.)				
	6	11	16	19	23
	mV×10⁻³				
11/14/57.....	56	94	82	123	140
12/17/57.....	42	51	73	77	89
1/8/58.....	51	58	123	130	152
5/1/58.....	61	68	83	114	106
6/23/58.....	65	71	96	91	99
7/25/58.....	67	67	100	105	129

* Subject MLE listed in Table 2. Each value represents the average of six or more frames.

TABLE 2

ELECTRICAL ACTIVITY (IN MV) OF LEFT MASSETER MUSCLE AT VARIOUS ORAL FORCES EXERTED AT FIXED BITE OPENING OF 6 MM.

SUBJECT*	FORCE (LB.)				
	6	11	16	19	23
	mV×10⁻³				
SL (5).....	42	75	92	97	115
LB (2).....	56	106	110	128	143
RM (3).....	196	258	228	272	279
JB (4).....	67	71	79	101	104
GD (3).....	90	121	136	144	161
MLE (6).....	57	68	93	106	119

* The number in parentheses refers to the number of different record series taken from the subject, and the mV values represent the average of these series.

categories other than neutroclusion. On this basis, no comparison could be made. A comparison of displacement (0.2–1.5 mm.) to no displacement (less than 0.2 mm.) showed a marked difference, as can be seen in Figure 4. However, the differences are not statistically significant. In Figure 5 it can be seen that the electrical activity of the joint-interference group was less than that of the group without interference. Computation of *t*-values indicates that the differences were significant ($P = 0.05$) when the force exceeded 11 pounds.

DISCUSSION

Gross electromyographic pattern differences under various conditions[1,2,5] or qualitative comparisons of amplitude versus time[3,4] have failed to produce reference base lines

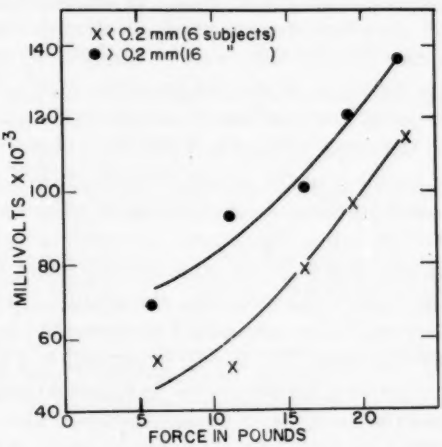

FIG. 4.—The electrical activity of the left masseter muscle in subjects with anterior displacement

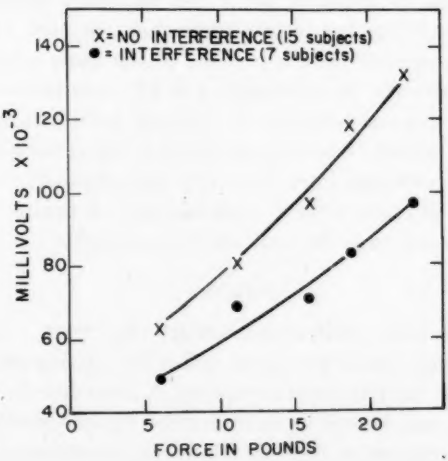

FIG. 5.—The electrical activity of the left masseter muscle in subjects with temporomandibular joint interference.

for definitive interpretations. Studies by Zwemer,[7] utilizing the mandibular isometric technique of Carlsöö,[8] suggest that potential amplitude is the prime factor for the interpretation of electromyograms. This is further demonstrated by the data of Geltzer.[9] The latter investigator's approach most nearly coincides with ours, in that he considered average "spike" magnitude (temporal muscles) versus occlusal force (20–200 lb.). However, Geltzer utilized a gnathodynamometer-type pressure device. Thus at least three variables were present: a change in pressure, a change in length of muscle, and a change in temporomandibular articulation.

In the data presented here the use of the bite block arranges the oral structures so that the only variable present when recording is the occlusal force. That variables in recording technique may be present is indicated by the data in Table 1. The variation at different recording intervals may be due in part to the difficulty in reproducing the geometrical placement of the electrodes and/or a variation in the electrode-skin contact. We have some evidence that the latter may be involved, in that the interelectrode resistance was not always the same. It was not practical, with our present equipment, to evaluate this factor properly and to adjust it accordingly.

Records were made on the subjects listed in Table 2 because of their availability for multiple recording. The variation in the values could have been due to differences in occlusion as well as to other factors indicated previously. The fact that the mandible was in the protruded position during the process of recording undoubtedly influenced the nature of the record, and this in some unknown way might have caused some of the variability observed in Table 2. Nevertheless, the relationship between the occlusal forces and muscle activity was what one would theoretically expect in view of the known facts about muscle function. This is well illustrated in Figure 4.

The lack of significance between the two groups in Figure 4 is not surprising, in view of the data in Table 1 and the small displacements involved. The difference associated with joint interference (Fig. 5) would be more acceptable with a larger group, and inferences must be made with caution.

Expressing the results in terms of the actual millivolts developed by the muscle under definite, fixed conditions has certain advantages; namely, (1) quantitative comparisons can be made between various muscles of the same subject, or from subject to subject, or from laboratory to laboratory, and (2) quantitative evaluations can be made of subjects with normal occlusion or occlusal disturbances. The data indicate that electromyographic activity can be quantitated in the fashion described here. However, evaluation of the variables introduced into electromyography must be made before the "normal" can be characterized. This has been recognized by others[10,11] interested in providing a sound basis for oral electromyography.

SUMMARY

By means of a single-beam oscilloscope, surface electrodes, a bite device to fix the bite opening (6 mm.) and record pressures, and a 16-mm. camera, electromyographic records were taken from the left masseter muscle. A procedure is described for quantitatively expressing the muscle activity in millivolts. Measurements were reproducible, once the electrodes were placed in position. However, variations in the level of activity occurred after the electrodes were removed and replaced. Regardless of the level of

activity, the nature of the response in all cases was similar; i.e., increasing bite forces (0–23 lb.) were related directly to muscle activity (0–279 mV $\times$ 10^{-3}). A difference in muscle activity was evident in subjects manifesting a slight temporomandibular joint disturbance or anterior displacement (0.2–1.5 mm.) of the mandible when compared with subjects with no joint disturbance or no anterior displacement, respectively.

We are indebted to Dr. Gordon Schoepfle, Department of Physiology, Washington University Medical School, for advice and the loan of the oscilloscope used in these studies; to Mr. S. Levitt and Mrs. M. L. Ellis for their technical assistance; and to Mr. R. W. Buchanan for construction of the "bite block."

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Morphologic Study of Oral Connective Tissues in Lathyrism

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INTRODUCTION

The subject of lathyrism has aroused considerable interest during the past few years. Investigation of the metabolism of connective tissue by pathologists, endocrinologists, and biochemists has been centered mainly upon experimental lathyrism. Knowledge of the disease termed "lathyrism" is important for two main reasons. First, it gives insight into the connective tissue aberrations produced by feeding rats with the seeds of *Lathyrus odoratus* (sweet pea), producing lesions similar to, if not identical with, certain idiopathic diseases of human connective tissue for which the etiology is unknown. Second, it has been relatively simple to produce alterations in the ground substance of connective tissue due to the feeding of *L. odoratus* seeds. Disturbances of connective tissue function may be associated with alterations such as occur in lathyrism, and the role that these disturbances may play in the production of symptoms and pathologic changes in connective tissue metabolism needs illumination.

The investigation to be described is an attempt to isolate some of the pathologic mechanisms that can be shown to alter metabolism of connective tissue. Two objectives were prominent in initiating this work: to investigate variations of the histopathologic and cytopathologic pattern of lathyrism and to gain whatever insight could be afforded concerning the nature of lathyrism.

REVIEW OF LITERATURE

The prominent role played by connective tissue in the lesions of lathyrism was recognized by the earlier investigators of this complex disease.[1–16] However, it is even more interesting that, within the specific lesions in the periodontal ligament, periosteum, and other mesodermal tissues, the connective tissue shows some features that in the light of recent knowledge may be considered suggestive of some relation to hyaline, amyloid, collagen, fibrin, and fibrinoid. In lathyrism the fibrous tissue is very great, and the cells are separated by tracts, lakes, or bodies of firm, imperfectly fibrillated tissue. In most areas of the periodontal ligament this tissue has the same vitreous appearance as that mentioned as being conspicuous in hyaline degeneration or necrosis, amyloid, fibrinoid, and collagen degeneration.

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The hyaline structure of areas of connective tissue in the periodontal ligament and the proliferation and differentiation of fibroblasts bordering these areas tend to explain the formation of the material as collagen. This led naturally to the question as to what precise relationship this process might have to the phenomenon of serous inflammation and to the formation of those poorly defined materials that are of great interest to connective tissue investigators, namely, "hyaline," "fibrinoid," "sclerosis," "amyloid," and "paramyloid."

The literature concerning these substances is extremely voluminous because of their implication in the disorders grouped by Klemperer *et al.*[18] and others[19] as "collagen diseases."

It has been observed that an alteration within the interstitial spaces known as "fibrinoid degeneration" occurs in a wide variety of conditions. It is apparent that the only anatomic requirement for definition of fibrinoid material is its extracellular location. Morphologically, it has been defined as homogeneous, bandlike, and highly refractile. Lillie[20] apparently favors the view that the term has little usefulness because of the present unsettled state of knowledge concerning the structures so designated.

It appears established, however, that, wherever found, fibrinoid material contains protein with a high arginine content. A strong periodic acid–Schiff reaction for polysaccharides has also been a constant finding. Metachromatic properties may or may not be present.[21] In a temporal, spatial, and configurational sense, Altshuler and Angevine[17] have emphasized that fibrinoid material can usually be shown to occur subsequent to, and in the region of, excessive accumulations of metachromatic ground substance, or acid mucopolysaccharides.

Theoretically, an analogous process occurs in normal collagen formation, the increased mucopolysaccharide in this case being provided chiefly by proliferating fibroblasts rather than by the process of serous inflammation,[22] which is supposed to admit polysaccharide to the site.

When scar formation occurs without significant fibroblastic proliferation, the end result is termed "sclerosis." When scar formation occurs in conjunction with abundant fibroblastic activity, the end result may be identical with advanced sclerosis; however, the process is referred to as "hyalinization" of the scar.

The concept is further being entertained that secondary amyloid deposition may occur purely as a result of a combination of protein (globulin) with relatively small quantities of polysaccharide. Primary amyloid, having a different distribution from the secondary type, is thought to be more closely related to the phenomenon of fibrinoid formation, inasmuch as it can sometimes be demonstrated to lie in a metachromatic matrix with toluidine blue.

Here the question as to the definition of paramyloid arises. This term has been used by Apitz[24] to designate specifically the amyloid occurring in association with multiple myeloma.

Although the above presentation of concepts is oversimplified, it should serve to explain interest in the very peculiar connective tissue found in the lesions of lathyrism.

MATERIALS AND METHODS

This study represents an investigation chiefly in histochemical and microscopic morphology. Repeatedly in the history of lathyrism and related disorders, more has

been expected of and claimed for morphologic methods than the methods could reasonably be expected to give.

Ninety-six male albino weanling rats, 45–50 gm. in weight, of the Holtzmann-Sprague-Dawley strain, were used in this investigation. The rats were divided into groups at weaning and placed on various diets containing *L. odoratus*. An additional group of male rats was pair-fed and served as normal littermate controls. Each of the experimental rats was paired with one of the controls of the same weight. The rats were housed in an open-bottom mesh cage. All control animals were sacrificed at the same time of day as the experimental animals. The rats were separately caged in an isolated room maintained at the usual laboratory temperature (70°–80° F.). The animals were weighed periodically, and their weight gains were recorded. Immediately after death the rats were autopsied. The carcasses were skinned and eviscerated. The skulls were removed, radiographed, and finally fixed in Zenker-formalin solution for 1 day or in Bouin's solution for 2 days.

Ground L. odoratus diets.—The experimental diets were as follows:

1. *Lathyrus odoratus* meal 50 per cent, casein 10 per cent, corn starch 25 per cent, sucrose 5 per cent, brewer's yeast powder (dried) 4 per cent, salt mixture[25] 4 per cent, and cod-liver oil 2 per cent.

2. *Lathyrus odoratus* meal 30 per cent, casein 10 per cent, corn starch 45 per cent, sucrose 5 per cent, brewer's yeast 4 per cent, salt mixture[25] 4 per cent, and cod-liver oil 2 per cent.

The *L. odoratus* diets were prepared by grinding the whole dry peas to a fineness permitting all the pea meal to pass through a 24-mesh sieve. The dry ingredients were mixed separately and stored, the cod-liver oil being added to portions of the mixture as needed.

The control diets were as follows:

1. This control diet contained the same ingredients as in diet 1 of the experimental diets but with the *L. odoratus* meal replaced by a 50 per cent dried and ground commercial edible pea meal.

2. Milled Rockland rat diet.

For the 50 per cent experimental sweet-pea diet, the feedings were continued for 3 weeks. The 30 per cent sweet-pea diet was fed for 8 weeks. Diets and distilled water were offered *ad libitum*.

It was known from pilot studies that rats fed a given percentage of sweet peas in their diets will continue to survive only a limited period of time. Therefore, the rats were sacrificed in advance of this period so that the histologic sections from the experimental groups would be uncomplicated by drastic terminal retrograde changes. Therefore, all rats developed lathyrism but were still active.

Histologic preparation and analysis.—After sacrifice and fixation, the upper and lower jaws were dissected free from the skulls, so that the tissue sections consisted of upper molars, upper incisors, lower incisors with lower molars, and the ramus and mandibular articulation. After 24 hours in the fixative, the tissues were decalcified with ethylenediaminetetraacetic acid (EDTA), which has a pH of 7.0. All material was decalcified for 6 days in EDTA. The tissue sections were double-imbedded separately with celloidin and paraffin in individual blocks. Mesiodistal sections through two buccal roots of the upper and lower molars, sagittal sections of incisors, and frontal sec-

tions of intact hemisections of skulls were prepared. All histologic sections were cut consecutively at 6–8μ. After mounting in Canada balsam, the cover slips were weighted down to obtain flat sections.

The histochemical stains and staining methods were as follows:

1. Harris hematoxylin and eosin Y.
2. Toluidine blue, 0.02–0.05 per cent in citrate buffer (pH 4.0).
3. Mallory's phosphotungstic acid–hematoxylin.
4. Periodic acid–Schiff (PAS). McManus' (1948) method, without using the acid-reducing rinse of Hotchkiss (1948). Collagen stains a yellowish pink, reticulin bright red.
5. Crystal violet for amyloid. Sections were stained in 0.1–0.2 per cent solution of crystal violet for 10 minutes. Amyloid is purple-red, background blue.
6. Van Gieson's 1 per cent aqueous acid fuchsin, 15 cc.; saturated aqueous picric acid, 50 cc.; distilled water, 50 cc.
7. Wilder's reticulin without counterstain, or Gomori's silver stain for reticulin without counterstain.
8. Masson's trichrome stain for collagen.
9. Aldehyde fuchsin after peracetic acid oxidation. This is a new stain for connective tissues including the periodontal ligament developed by Fullmer and Lillie.[29]

Tendons, ligaments, and periodontal ligaments consist almost entirely of collagenous fibers and ground substance. When sections of these tissues are subjected to the staining procedure given below, some fibers, previously described as collagen, *react with the purple stain*, while others remain unreactive and are colored by the counterstain. Mesodermal tissues subjected to stress apparently contain or develop aldehyde fuchsin–peracetic acid-positive fibers demonstrated by this stain.

Analysis of staining methods.—Briefly, the reasons for using the above methods are as follows:

1. The Harris hematoxylin-eosin Y stain is used so widely in investigations of this kind that little need be said of it here.

2. Toluidine blue with metachromasia is at present believed to demonstrate the presence of polymerized acidic substances. When observed in connective tissues, metachromasia is considered presumptive evidence of the presence of considerable quantities of acid mucopolysaccharides. The importance of the sulfate radicals in producing toluidine blue metachromasia in the connective tissues is as yet not clear; however, this method is used only for the demonstration of "ground substance," without implications as to the specific chemical composition of this material. Ground substance for the purposes of this investigation is defined as extracellular material containing highly polymerized glycoproteins.

3. Mallory's phosphotungstic acid–hematoxylin staining, while still an empirical method, is useful in identification of fibroglia and fibrin, both of which give a dark-blue reaction and are differentiable on the basis of structure and anatomic location. Fibroglia are generally relatively straight, uniform, and of moderately heavy caliber, while the fibrils of fibrin are more delicate, irregular, and tend to form reticular structures. The Mallory phosphotungstic acid stain also characteristically stains collagen and reticulum in various shades ranging from yellow to red-orange or merely red. With the method employed, amyloid reacted with a pink-orange or deep salmon color.

4. The periodic acid–Schiff method is widely known as a method of demonstrating carbohydrates in tissues, but it is also recognized that conjugated proteins and lipids (e.g., cerebrosides) will give a positive reaction. The method is considered to demonstrate the presence of 1–2 glycol linkages, occurring in a wide variety of compounds. In its employment in this investigation, the PAS reaction is interpreted to imply the presence of carbohydrate when such an interpretation is consistent with the results of other procedures on the same structures or with existing knowledge of a carbohydrate component of a particular structure, such as ground substance or collagen.

5. The crystal violet method is a standard procedure for identifying amyloid, that typically reacts with a purple-red metachromasia.

6. The Van Gieson procedure was used for its value in identifying structures reacting with the characteristics of collagen (red). Muscle and cornified epithelium are yellow; nuclei blue to black. With this stain, amyloid, fibrin, and "fibrinoid" give a yellow color, although Altshuler and Angevine[17] have reported a red reaction in material they found positive for fibrinoid.

7. Wilder's reticulum stains collagen a rose color and reticulum fibers black. Gomori's silver-precipitation method for "reticulin" was used for the purpose of correlating "reticulin" with the formation of connective tissue in lathyrism. This method is also empirical and does not permit sharp differentiation of "reticulin" from collagen, if, indeed, any actual chemical difference exists. However, dense deposits of collagen give a reddish-brown-toned effect with this method, while more delicate fibers are intensely black.

8. Masson's trichrome stain for collagen gives the following results: nuclei stain black; cytoplasm, keratin, muscle fibers, and intercellular fibers stain red.

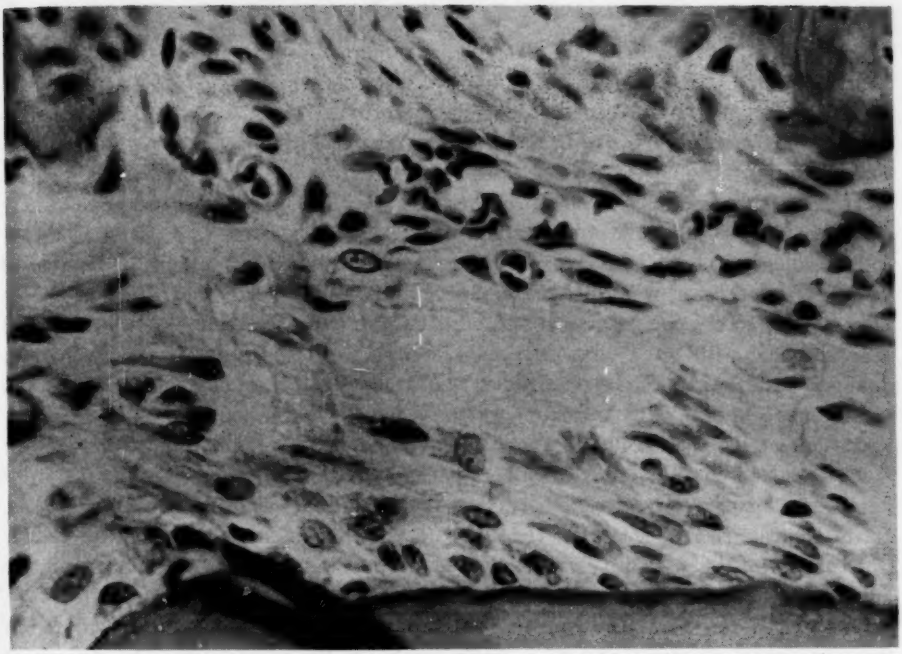
9. When aldehyde fuchsin was used after peracetic acid oxidation for the presence of fibers in periodontal ligaments, many fibers of the periodontal membrane stained a brilliant purple; others remained unstained. The fibers remaining unstained are believed to be collagen, and the purple-stained fibers are believed to be a stress fiber.

MORPHOLOGIC RESULTS

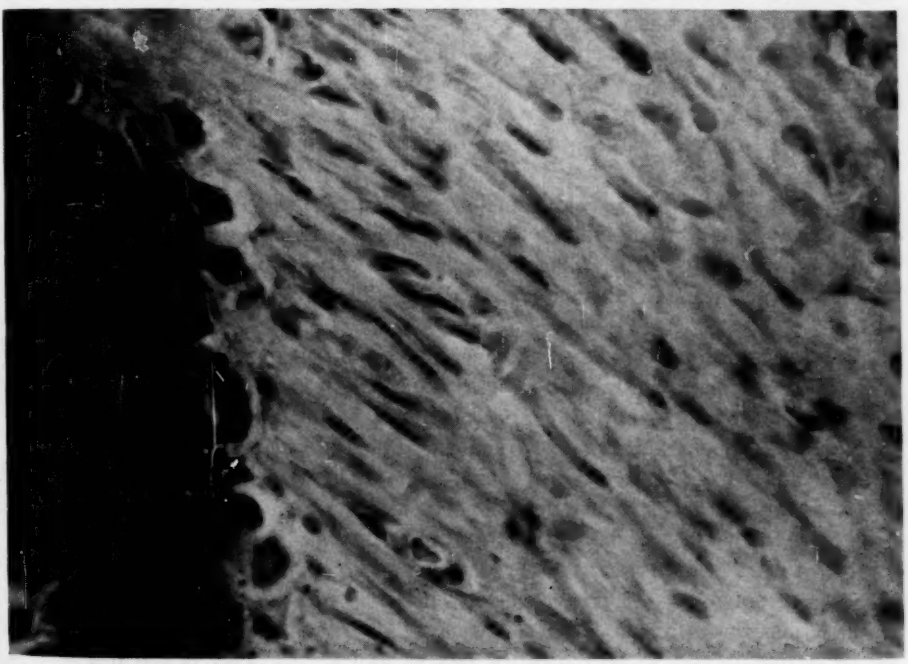
The animals examined provided a spectrum of the connective tissue changes in lathyrism. Among them were examples in which virtually no excessive connective tissue formation after short periods of feeding *L. odoratus* was found. Other examples showed the presence of large quantities of hyaline eosinophilic material; still others exhibited intermediate degrees of connective tissue formation. Obvious fibroblastic activity was present in some, while others showed the lack of fibroblastic participation. The deposits were stained by the procedures previously described under "Materials and Methods."

The various elements concerned in connective tissue formation, together with possibly related substances such as amyloid, fibrinoid, and hyaline, will be considered individually so as to maintain as sharp a distinction as possible between them. It will assist in that effort, first, to provide a general description of various changes in lathyrism as they were seen in hematoxylin- and eosin-stained material.

It was observed that moderate to large amounts of connective tissue were present in the lesions of lathyrism (Fig. 1). Along with this connective tissue the presence of



A



B

FIG. 1.—*A*, Photomicrograph of a mesiodistal section of the apical third of the periodontal ligament of a male rat fed a 50 per cent sweet-pea diet for 3 weeks. Notice the homogeneous lathyritic body in the center of the ligament. Principal collagenous fibers of the periodontal ligament are masked as they enter this acellular and avascular mass. Hematoxylin and eosin stain; orig. mag. ×770. *B*, Control ligament.

significant amounts of fibroblastic activity was evident in the periosteum and periodontal ligament. In most cases fibroblasts were extremely numerous within the periosteum at sites of tendinous and ligamentous insertions. In the periodontal ligament, differentiation of fibroblasts occurred, with cells increasing in size, as well as showing an increase in the basophilic staining of their cytoplasm. The enlarged fibroblasts were arranged in a palisade manner into rows.

Regardless of whether fibroblastic activity consisted of an increase in the number of cells (periosteum) or of an increase in size and basophilia (periodontal ligament), the formation of connective tissue was most abundant in the periosteum, in the periodontal ligament, and within the temporalis muscle close to its insertion. However, in most advanced cases, areas of the periosteum were transformed into exostoses consisting of spongy bone trabeculae or intramembranous bone. In the periodontal ligament transformation occurred into hyaline structures composed of an amorphous eosinophilic material surrounded by highly basophilic palisading fibroblast-like cells. The definitive lesion consisted of rows of cells between large hyalinized bodies of connective tissue. It also appeared that the greater the degree of fibroblastic activity present, the larger were the deposits of relatively acellular connective tissue in the zones immediately adjacent to the palisading fibroblasts.

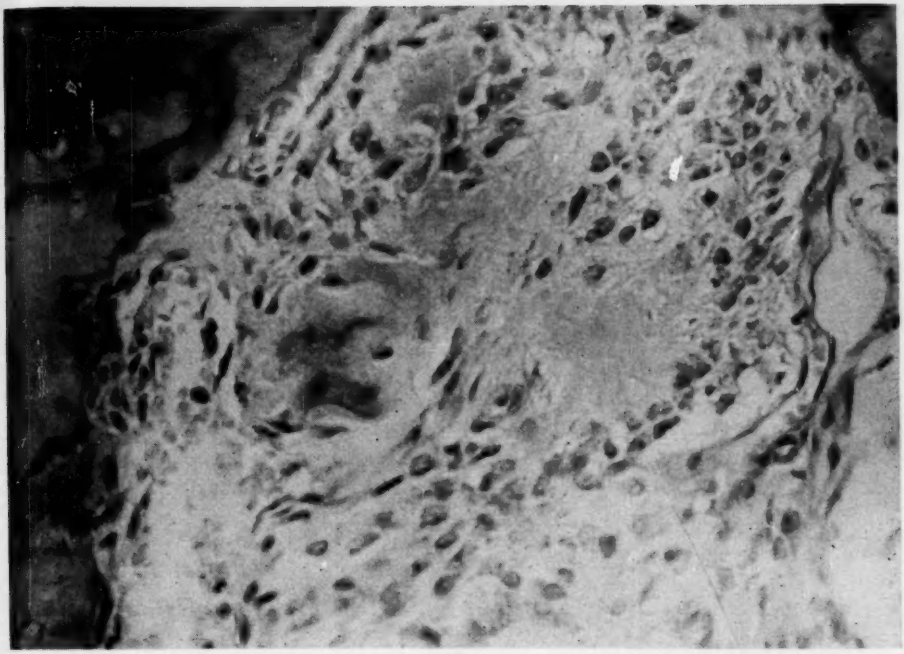
Of special interest in lathyrism were the deposits of eosinophilic hyaline material in the periodontal ligament and to a lesser extent in the periosteum. The deposition appeared to represent a swelling and an increase in the non-cellular reticulum of these areas of connective tissue. The deposits in the periodontal ligament were often so homogeneous as to have the appearance of a proteinaceous transudate, fixed *in situ* (Fig. 1). They were very similar in appearance to amyloid. The hyaline deposits throughout the periodontal ligament were so formed as to suggest that the collagenous fibers had undergone marked swelling. Eosinophilia was extremely intense in these deposits.

It was possible, within a given animal as well as from one animal to another, to find progressive degrees of eosinophilic hyaline material as described above, ranging from moderate thickening of the collagen after 1 week of *L. odoratus* feeding to massive hyaline fibrosis occupying the majority of the width and length of the periodontal legament.

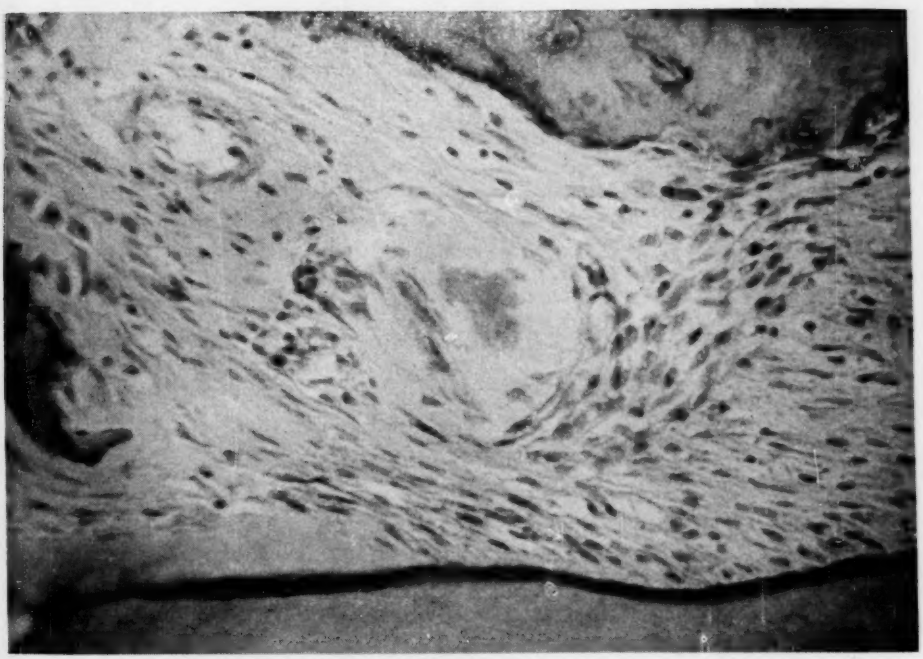
With hematoxylin and eosin stain, minimal to no fibrous internal structure appeared to be a development within such deposits, consisting of a few fine fibrils having no definite orientation. The fibrils were masked and imbedded in abnormal amounts of an amorphous eosinophilic material surrrounded by palisading fibroblast-like cells.

In advanced cases there was a tendency for a mineral-like process of precipitation to occur in the internal structure of the hyaline deposits. Such areas appeared similar to dystrophic calcification occurring as corpuscles of incipient calcification (Fig. 2).

A point of special interest was the fact that, in the periodontal ligament showing the greatest amounts of hypocellular connective tissue deposits, an absence of plasma cells in the lesions was also seen. In fact, plasma cells were not present at all in the periodontal ligament; thus in lathyrism the lack of any amyloid-plasma cell relationship tends to aid in ruling out amyloid as the amorphous material. This was not an observation limited to only a single and, therefore, possibly exceptional case but one that has



A



B

FIG. 2.—*A, B,* Photomicrograph of a mesiodistal section of the gingival third of periodontal ligament near the alveolar crest of a male rat fed a 50 per cent sweet-pea diet for 3 weeks. Notice the presence of two spherical lathyritic bodies, one showing an affinity for incipient calcium-like salt deposition. In addition to dystrophic-type calcification, a few cells are also included in the lathyritic bodies. Hematoxylin and eosin stain; orig. mag. ×670.

been consistently noted in the histologic examination of lathyrism where this type of connective tissue alteration was prominent.

Eosinophilic, hyaline-like material had been noted to occur in two anatomic locations in lathyrism. In both, the formation of this material appears related to degeneration or eosinophilia. The difference between the two depends on the cellular elements present and upon the quantity of eosinophilic homogenous material. In the one instance, small homogeneous eosinophilic deposits were found in foci of young proliferating connective tissue of the periosteum, where it is the first sign of bone formation with the appearance of a hyalinized, homogeneous intercellular substance around cells of the young mesenchyme. The fibrils or fibers present seemed to disappear, while the cementing substance, originally semifluid in character, seemed to have undergone a change in consistency. The collagen molecules of the depolymerized fibrils and the highly polymerized glycoprotein of the cementing or ground substance formed at this time a homogeneous substance appearing eosinophilic that resembled hyaline. Large numbers of closely packed fibroblasts were present, having basophilic cytoplasm, dense nuclear membranes, coarse chromatin network, and eccentric nuclei with prominent nucleoli. In some cases the palisading arrangement of cells, so well demonstrated in the periodontal ligament, could also be seen in the periosteum. Mitoses were frequent only in the periosteum.

Furthermore, very similar deposits of homogeneous, eosinophilic material were found in the periodontal ligament forming a sort of matrix in layers between rows of cells where large osteoblast-like cells occurred in especially heavy concentrations. It was noted that the fibroblastic proliferation and concentrations of cells were consistently situated adjacent to this eosinophilic material. The material was, in fact, surrounded by numbers of osteoblast-like or fibroblast-like cells, as though these cells in such situations represented foci of persisting periodontal tissue, about to be replaced by the specific lesions of lathyrism. It should be noted that the osteoblastic- or fibroblastic-like collections of cells adjacent to the eosinophilic material do not have the characteristics of fibroblasts of normal periodontal ligament (Fig. 3). It appears that cellular breakdown may have occurred in aggregates of connective tissue cells forming the eosinophilic hyaline material.

Consequent to the special staining and histochemical studies, the various connective tissue elements described above have been classified and will be correlated below under the appropriate headings.

Ground substance.—As a generality, it was observed that areas of stress showed the most reactive sites. The ground substance was present in greatest quantities in those tissues in which fibroblasts were most actively dedifferentiated, enlarged, and numerous. Furthermore, the demonstrable ground substance was usually located within the fibrillar meshes of connective tissue containing readily demonstrable or enlarged fibroblasts. The largest amount of ground substance appeared in the periodontal ligament, with comparable but lesser amounts also present in the more cellular bands of fibrous tissue traversing the widened periosteum at sites of muscle insertions and in the cellular connective tissue replacing portions of the temporalis muscle. Cells apparently of fibroblastic or osteoblastic identity could always be found in these areas. Dissociation of metachromasia from fibroblasts was possible, since no metachromasia could be demonstrated in the eosinophilic areas (Figs. 5 and 6), though we were readily im-

pressed by the great disproportion of ground substance as well as of collagenous connective tissue of fibroblasitc activity in these areas. Ground substance always appeared to have an interesting relationship to the fibroblast- or osteoblast-like cells, although the presence of fibroblasts in the same area definitely does not permit one to conclude that the non-metachromatic material was of other than fibroblastic origin. An increase in acid mucopolysaccharide in the periodontal ligament may be provided chiefly by proliferating fibroblasts present in palisade arrangement and bordering the hyaline material. This view was opposed to the accumulation of polysaccharide by the process of serous inflammation. However, this was not tenable on the basis of the van Gieson reaction (Fig. 4), since appreciable quantities of acid mucopolysaccharide were not demonstrable, inasmuch as a red reaction developed instead of the bright blue for acid

FIG. 3.—Photomicrograph of a mesiodistal section of the periodontal ligament of a male rat fed a 30 per cent non-supplemented sweet-pea diet for 8 weeks. Notice the amorphous collagenous-like material of lathyritic bodies occurring in layers separated by palisade-arranged cells principally in the center of the ligament. Masson's stain; orig. mag. ×675.

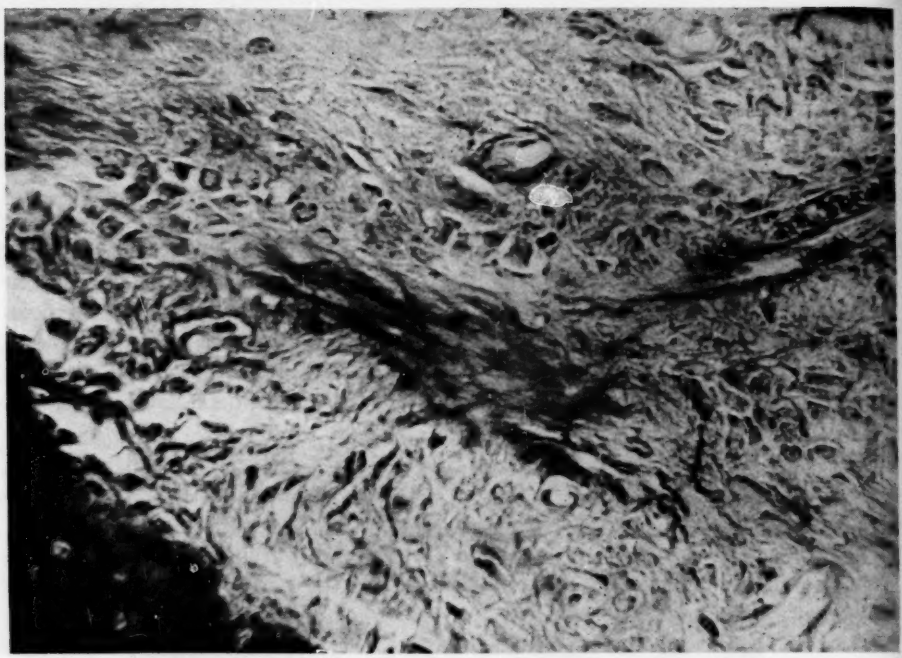


FIG. 4.—Photomicrograph of a mesiodistal section of the periodontal ligament of a male rat fed a 30 per cent non-supplemented sweet-pea diet for 8 weeks. Notice the abundant bundles of fibrillar collagenous material in the center of the lathyritic body of the ligament bordered by rows of fibroblasts. Van Gieson's stain; orig. mag. ×725.

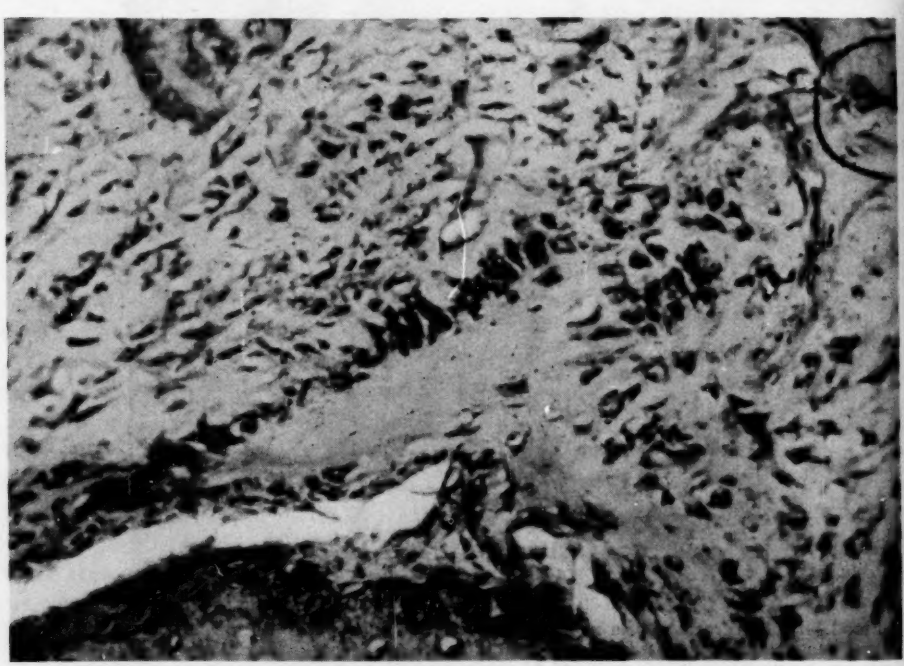


FIG. 5.—Photomicrograph of a mesiodistal section at the apex of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the homogeneous lathyritic body with palisaded fibroblasts. The amorphous bodies stain blue with toluidine blue and thus were not metachromatic. Toluidine blue stain; orig. mag. ×400.

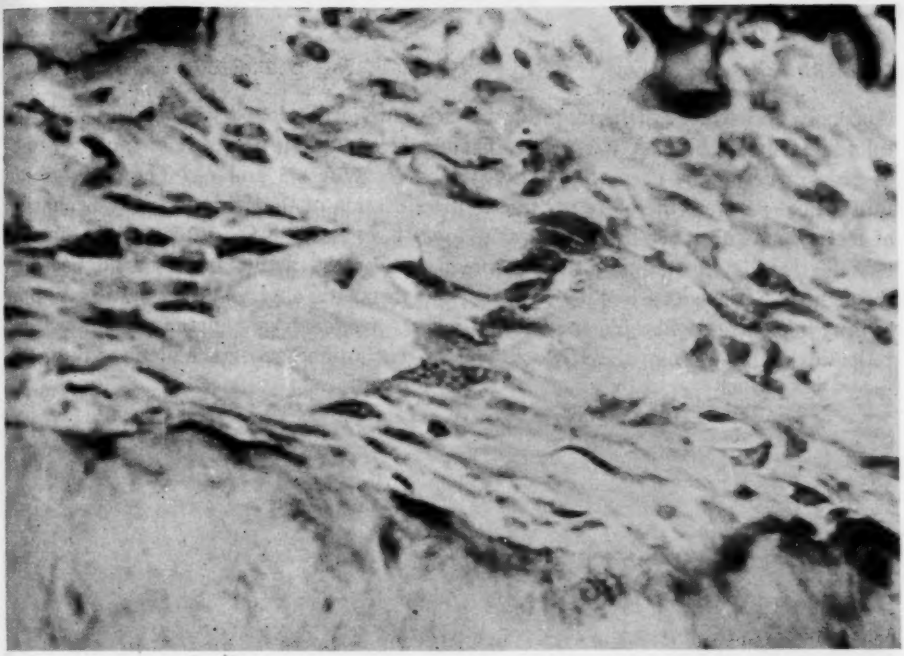


FIG. 6.—Photomicrograph of a mesiodistal section from the apex of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice that the lathyritic bodies stain weakly positive with crystal violet and were not metachromatic. Crystal violet stain; orig. mag. ×970.

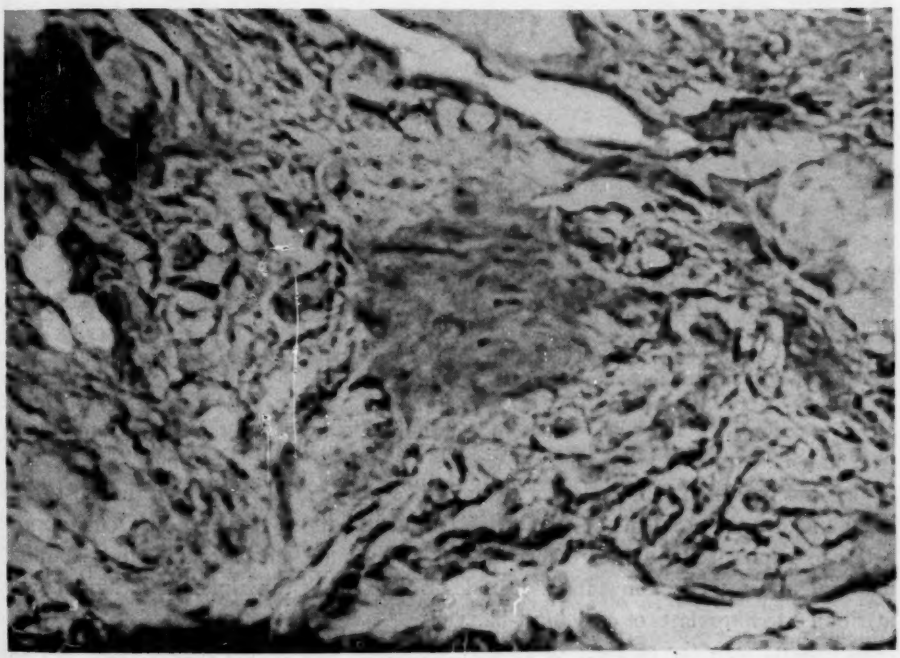


FIG. 7.—Photomicrograph of a mesiodistal section from the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the lathyritic body in the center of the ligament. It consists of an afibrillar homogeneous structure. The collagenous principal fiber bundles of the ligament lose their identity, becoming masked as they enter the lathyritic body. Mallory's phosphotungstic acid–hemotoxylin stain; orig. mag. ×890.

mucopolysaccharide. Furthermore, no metachromasia developed with crystal violet or toluidine blue; therefore, it appears that significant quantities of acid mucopolysaccharides may not be present in lathyritic bodies (Figs. 5 and 6). The eosinophilic lathyritic cores were therefore not due to precipitation of the acid mucopolysaccharide of the ground substance by its combination with an alkaline (basic) protein (Fig. 7).

Wilder's reticulin stain and silver impregnation by the method of Gomori of sections through these same locations showed non-metachromatic areas staining blue-gray, presenting a picture of fibroblasts inclosing layers of swollen and increased non-cellular reticulin (Fig. 8). From this it would appear that ground substance alone may give a

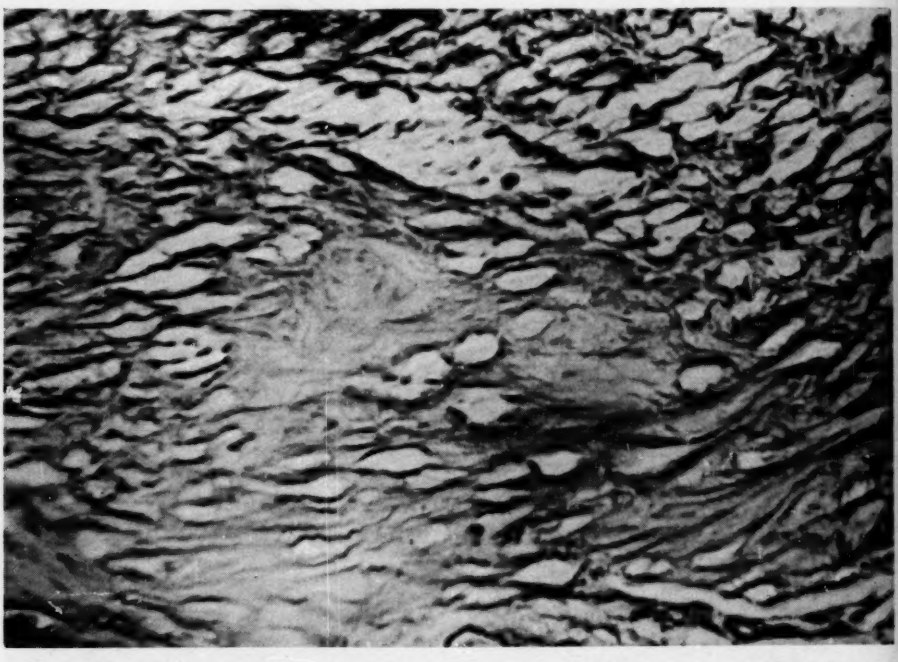


FIG. 8.—Photomicrograph of a mesiodistal section from the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the two lathyritic bodies with a loss of individual reticulum fibers as they enter the amorphous zones, becoming masked into a homogeneous structure. Wilder's reticulum stain; orig. mag. ×750.

silver reaction identical with "reticulin" or that reticulin may be formed from, or appear in concentrations of, ground substance.

It is not possible in the realm of this investigation to explore or solve the most vital and fundamental problems of connective tissue formation. However, it seems appropriate to emphasize that the presence of increased ground substance and silver-staining material under the circumstances just described during experimental lathyrism was attributed to fibroblasts or osteoblast-like cells.

In dealing with the problem of ground substance, one should realize that, because of its water-solubility, variations in fixation may affect its demonstration. Formalin, for example, was found not to be ideally suited to the study of this material. However,

formalin-fixed tissues compared well with alcohol-sublimate–fixed material in retention of lathyritic bodies in the present work. This fact raised the question as to whether the eosinophilic material observed may chiefly represent mucoprotein rather than mucopolysaccharide. The eosinophilic lathyritic bodies described above gave in all instances a weakly positive periodic acid–Schiff reaction (Fig. 9). There seems reason to doubt, therefore, that the material concerned should properly be designated "ground substance" regardless of current uncertainties as to its exact chemical nature.

Similar weak periodic acid–Schiff reactions have been recorded for collacin or collagen in the state of basophil degeneration. Thus the lathyritic body may be a form of abnormal or degenerated collagen (Figs. 10-13). The weak PAS reaction may be essen-

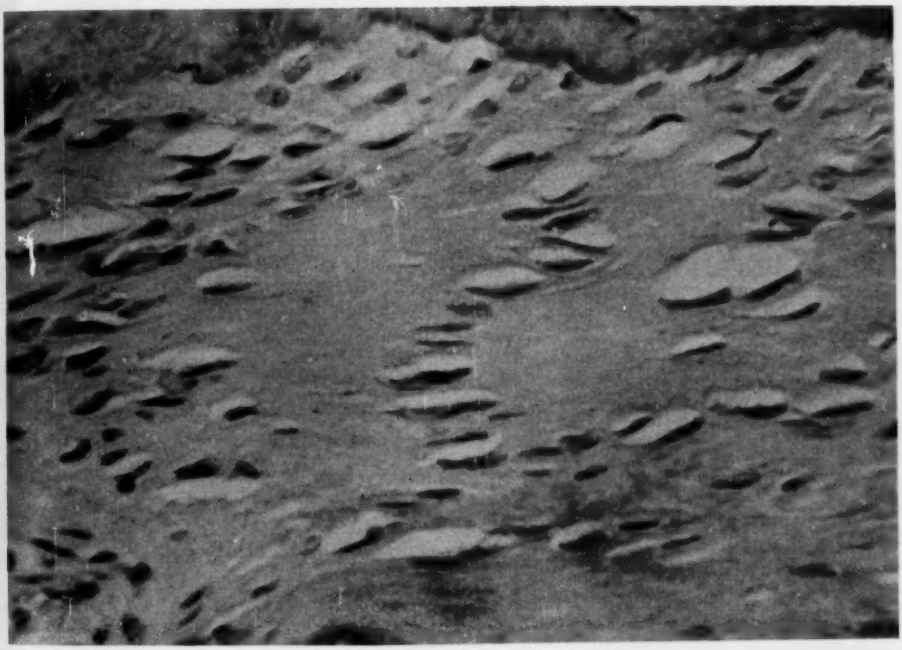


FIG. 9.—Photomicrograph of a mesiodistal section from the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the two lathyritic bodies in the center of the periodontal ligament showing a weakly positive staining reaction with periodic acid–Schiff stain; orig. mag. ×820.

tially due to the deposition of a very minimal polysaccharide-containing substance in the altered collagen produced by the initial lathyrism (Table 1).

Collagen.—The histologic definition of collagen is not clear, as evidenced by the discussion of Gersh's presentation[26] in the 1951 Josiah Macy conference on connective tissue. While certain features of collagen were considered "typical," such as a fibrous structure, a red reaction with Van Gieson's stain, blue with Masson's trichrome, red-orange with Mallory's phosphotungstic acid–hematoxylin, an absence of metachromasia with either crystal violet or toluidine blue, and a positive reaction with periodic acid–Schiff, however, one encounters connective tissue elements showing only some of these features (Table 1).

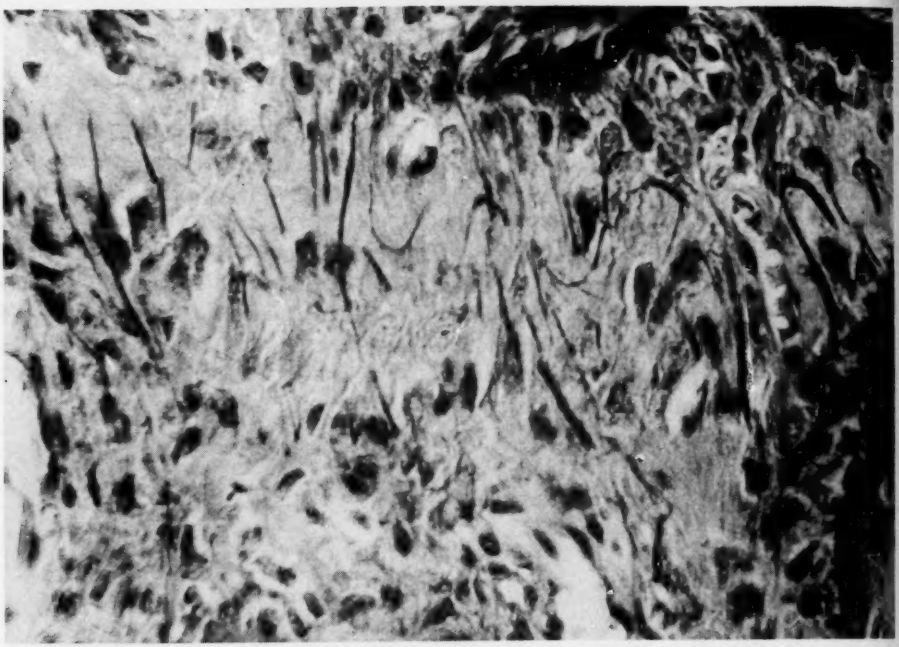


FIG. 10.—Photomicrograph of a mesiodistal section of the apical region of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice that in the region of the amorphous eosinophilic afibrillar lathyritic body are well-formed purple fibers of varying widths and lengths. They run in an unoriented and haphazard manner, frequently curving upon themselves and returning to the location from which they arose. Aldehyde fuchsin stain after peracetic acid oxidation; orig. mag. ×770.

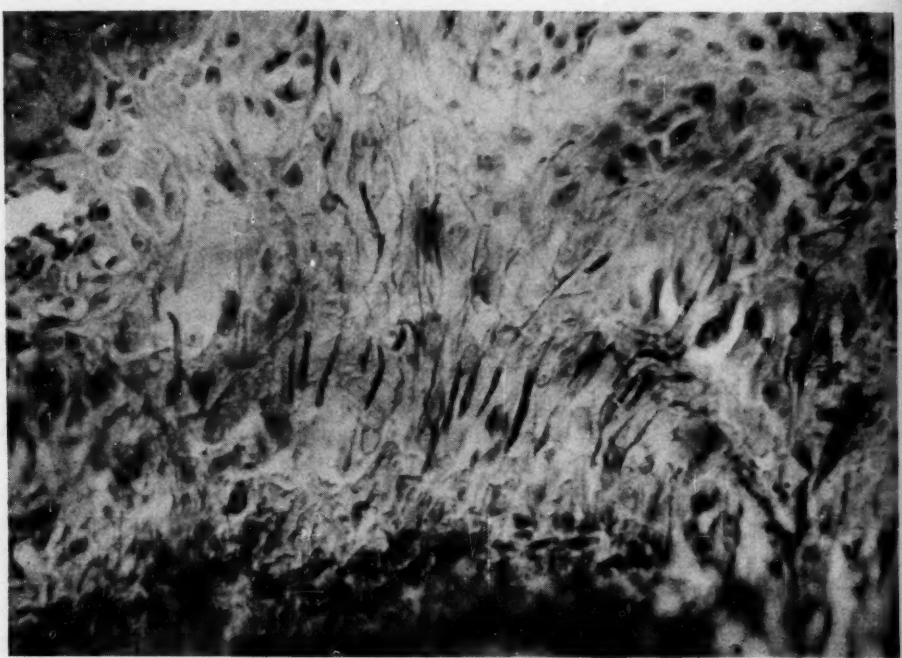


FIG. 11.—Photomicrograph of a mesiodistal section of the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the broad acid-resistant purple fiber that takes the aldehyde fuchsin stain after peracetic acid oxidation. The purple fibers traverse the amorphous eosinophilic lathyritic bodies. Purple fibers are neither collagen, reticulum, or elastic fibers. Aldehyde fuchsin stain after peracetic acid oxidation; orig. mag. ×475.

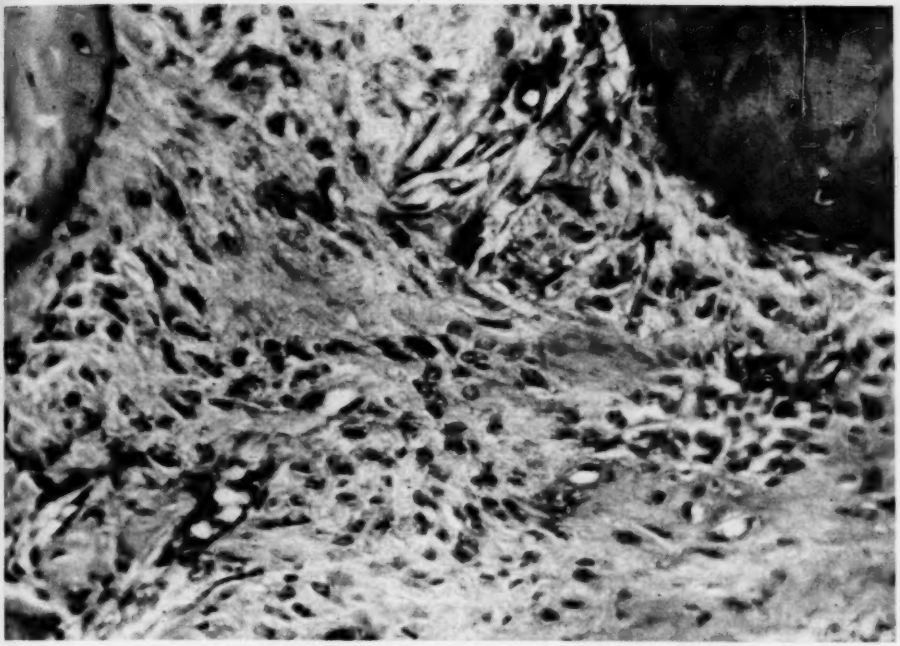


FIG. 12.—Photomicrograph of a mesiodistal section of the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the amorphous lathyritic bodies in the center of the ligament, showing an absence of the purple fiber so evident after peracetic acid oxidation. Aldehyde fuchsin stain without peracetic acid oxidation; orig. mag. ×500.

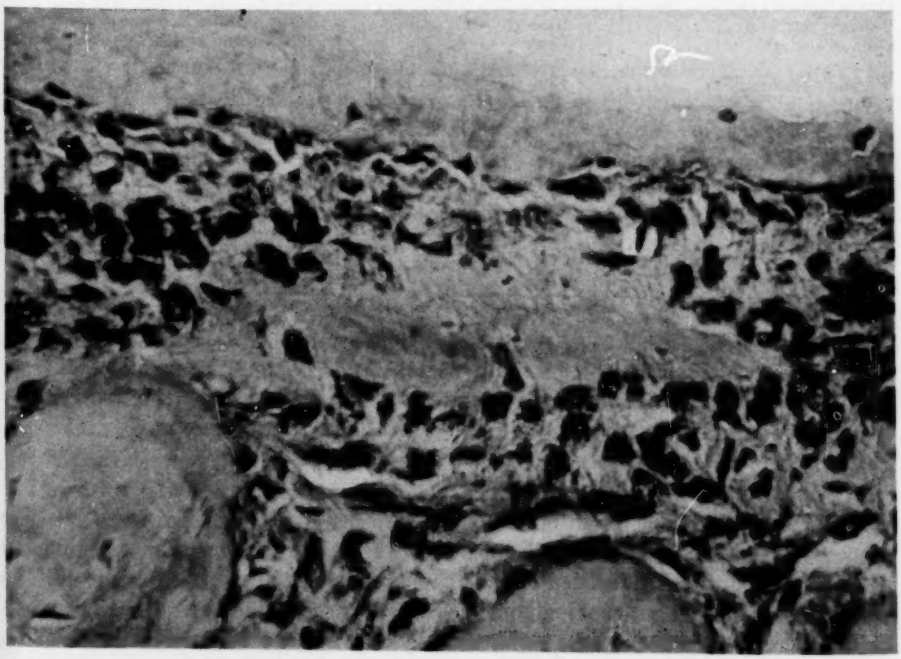


FIG. 13.—Photomicrograph of a mesiodistal section of the apical third of the periodontal ligament of a male rat fed a 30 per cent sweet-pea diet for 8 weeks. Notice the homogeneous avascular lathyritic body, giving the histochemical reaction for a collagen-like material with Masson's stain; orig. mag. ×770.

Thus, Gersh[26] referred to collagen without a fibrous structure as "poor collagen," while others wondered whether substances showing most, but not all, of the features of collagen should not be designated by another term.

In the present study, connective tissues of a hyaline nonfibrous nature were encountered. It therefore became necessary to make some arbitrary definitions in terms of the reactions used in this study:

Typical Collagen: Fibrous; internal structure, eosinophilic, pale, orthochromatic with toluidine blue and crystal violet, red with Van Gieson's stain, red-orange with Mallory's phosphotungstic acid–hematoxylin, moderately positive to periodic acid–

TABLE 1

HISTOCHEMIAL STAINING REACTIONS OF HOMOGENEOUS, TRANSLUCENT MATERIAL DEPOSITED IN PERIODONTAL LIGAMENT AND PERIOSTEUM OF MANDIBLE

Histochemical Stain	Reactions of Eosinophilic Areas	Histochemical Reactions for Normal Collagen
Perio ic acid–Schiff	Light pink, weakly positive (Fig. 9)	Positive, red-purple to light pink or rose
Van Gieson	Red (Fig. 4)	Collagen: red, amyloid, fibrin, and fibrinoid-yellow; acid mucopolysaccharide, bright blue
Toluidine blue	Blue, absence of metachromasia; no acid mucopolysaccharide (Fig. 5)	Absence of metachromasia; metachromasia, pink
Harris hematoxylin and eosin Y	Light pink (Fig. 1)	Pink
Wilder's reticulin	Blue-gray (Fig. 8)	Collagen and reticulum black
Gomori's silver	Gray	Collagen and reticulum black to red-brown
Masson's trichrome	Blue (Figs. 3 and 13)	Blue
Crystal violet	Absence of metachromasia; no acid mucopolysaccharide (Fig. 6)	Absence of metachromasia; amyloid, purple-violet or purple-red
Mallory's phosphotungstic acid–hematoxylin	Red-orange (Fig. 7)	Collagen red-orange; fibrin, deep blue
Aldehyde fuchsin after peracetic acid oxidation, in periodontal ligament	Purple fiber in eosinophilic bodies (Figs. 10, 11, 12)	Pink collagen; purple-resistant fiber to BAPN

Schiff reaction, reddish-brown with Wilder's stain and Gomori's silver impregnation, and blue with Masson's trichrome stain.

Hyaline Non-fibrous Collagen: Characteristics identical with the above except for the absence of a fibrous structure detectable with the light microscope (Fig. 1).

DISCUSSION

Hyaline non-fibrous collagen made up the majority of the proliferating connective tissue present in the periodontal ligament and of the periosteum in all experimental animals. Indeed, in consideration of the anatomic likeness of many of the deposits in lathyrism to fibrinoid and amyloid, one may be surprised to meet with evidence showing that these deposits were preponderantly hyaline non-fibrous collagen. In addition to characteristic staining reactions, they can be shown to have a non-fibrous structure for the most part, although within the mass of non-fibrous collagen one consistently

finds foci of narrow to wide purple fibers with aldehyde fuchsin stain after peracetic acid oxidation, traversing between fibroblasts and extending across the lathyritic bodies. Variations in the quantity of this purple fiber occurred from deposit to deposit of the lathyritic collagen. Aldehyde fuchsin unoxidized sections did not show the presence of the purple lathyrus-resistant fibers. This fiber in lathyritic periodontal ligaments was not disintegrated by the BAPN* toxin. It thus resists destruction when exposed to BAPN.

Therefore, from the viewpoint of collagenous fibers and histochemical studies, this material may be classified as hyaline non-fibrous collagen. However, by means of the special histochemical stain, purple BAPN-resistant fibers (non-collagenous) were demonstrated with peracetic acid oxidation and aldehyde fuchsin stain in the periodontal ligament of lathyritic animals (Table 1).

The purple BAPN-resistant fibers traversed the periodontal ligament in a mesiodistal direction, as well as in an apicoincisal direction. They did not appear to be oriented uniformly as the principal collagenous bundles of this ligament (Fig. 10).

A point of special interest in lathyrism was that, although principal collagenous and elastic fibers readily undergo disintegration, the oxidized peracetic acid–aldehyde fuchsin-stained fibers of periodontal ligaments were completely immune.

Another point of some significance with respect to collagen was the presence in some sections of quantities of it occurring perivascularly. This was likewise considered a characteristic of amyloid. In the perivascular location of areas of hyaline eosinophilic material, no evidence was present to show that this material was the immediate result of fibroblastic activity, since it was impossible to find enlarged fibroblasts adjacent to vessels.

Whether these non-fibrous collagenous masses were laid down chiefly as part of an accretive process was difficult to determine. Since some of the deposits were apposed to capillaries or arterioles of the periodontal ligament, the acquisition of building material may, in part, come from the circulating blood. On the other hand, the majority of the free boundaries of these deposits were virtually lined by easily identifiable fibroblasts or osteoblast-like cells; thus the situation was analogous to the relationship of plasma cell myeloma, in which amyloid formation occurs with plasma cells lining free borders of the deposits.

Attempting to reconcile these two outstanding features—e.g., hyaline non-fibrous deposits and fibroblastic activity—the hypothesis was formulated that the collagenous deposits might result from, or were at least contributed in part by, a combination of a product carried by the blood stream with a product of fibroblast- or osteoblast-like cells. Such a mechanism would be in opposition to Rossle's concept[22] of sclerosis without fibroplasia as the end result of serous inflammation.

Non-fibrous hyaline collagen was found to occur at anatomic sites identical with those in which typical collagen should be formed. In particular, the periodontal ligament and widened fibrous periosteum were more likely to show a hyaline character.

In most instances where non-fibrous deposits existed, typical collagen could be demonstrated nearby, with a masking of the collagen bundles as they entered the non-fibrous eosinophilic bodies, suggesting a close, morphogenetic relationship of the two.

Amyloid and paramyloid.—No deposits of material giving a definite metachromatic

* BAPN = beta aminoproprionitrile present in *L. odoratus*.

reaction with crystal violet or toluidine blue were encountered in lathyrism, while tissues with amyloid give typical results. Van Giesen's stain failed to yield the yellow amyloid reaction (Table 1) in this investigation. With Masson's stain, the deposits yielded a typical blue color consistent with collagen (Fig. 3).

"Fibrinoid" and fibrin.—"Fibrinoid," or, more properly, fibrinoid material, is still poorly defined and understood. As already stated, opinion concerning this material ranges from complete skepticism as to the existence of an entity deserving the name to a liberal acceptance of the term to include eosinophilic deposits with many or few of the characteristics of fibrin, occurring in a wide variety of conditions.

Because of this situation, some arbitrary definition is necessary, and in the following account the materials referred to as "fibrinoid" are defined as showing all the staining and histochemical properties of fibrin.

Using this definition, it is implied that the material in lathyrism could be differentiated from fibrin. The grounds for supposing that the deposits to be described were not fibrin is that their anatomic location was different from locations where fibrin is commonly found and that their histochemical reactions proved negative for fibrin.

The characteristics of fibrinoid as defined above are as follows: highly refractile, irregularly bandlike eosinophilic material, generally forming coarsely reticular structure or solid masses in extracellular regions; not metachromatic with toluidine blue or crystal violet, moderately or strongly positive to periodic acid–Schiff reaction, yellow or pale orange with Van Gieson's stain, dark blue with Mallory's phosphotungstic acid–hematoxylin (PTAH) and black with Gomori's silver-impregnation method. The material stained negative for fibrin on the basis of the Van Giesen reaction, where a red result was evident instead of the yellow color typical of fibrin or fibrinoid (Fig. 4). The material similarly stained negative for fibrinoid on the basis of Mallory's PTAH, where a red-orange result occurred instead of deep blue (Fig. 7).

For the most part, there was no demonstrable metachromasia with toluidine blue in areas where fibrinoid-like material was present.

In the presence of large amounts of collagen the deposits of fibrinoid-like material were conspicuous in hematoxylin and eosin sections, but could also readily be found in Mallory's phosphotungstic acid–hematoxylin preparations, where they stood out as heavy red-orange densities. Since they failed to stain dark blue in the periodontal ligament, this tends to rule out the material as fibrinoid.

It seems indicated that in lathyrism, at least, the very extensive amount of connective tissue formation already described does not appear directly related to the fibrinoid material under consideration. Neither in staining reaction, extent, configuration, nor spatial relationships did the deposits of fibrinoid-like material seen in this study offer any suggestion that a really significant part of the connective tissue frequently associated with lathyrism was an end product of the fibrinoid alterations.

Cerainly there is no reason to draw hard-and-fast conclusions on the nature of lathyrism on the basis of the connective tissue changes described in this investigation. At best, these changes may collectively constitute a pattern which seems rarely, if ever, to be exactly duplicated in other conditions. Taken individually, however, these same changes are to be found in varying degrees in a medley of conditions ranging from normal wound healing, through various forms of infection and inflammation, including hypersensitivity states, to metabolic and, finally, neoplastic disease. The number of

ways that connective tissue may react in pathologic states is limited, and in lathyrism it appears that practically all these reactions may occur.

It seems permissible to take a special interest in the connective tissues of lathyrism on the basis of certain outstanding features such as the predominantly ligamentous distribution of collagenous deposits; the frequently striking correlation between the amount of collagen formed and the amount of fibroblastic activity evident; the anomalous histochemical reactions of some of the deposits, as well as their hyaline structure, suggesting features of amyloid in spite of predominantly collagenous characteristics; the association of "sclerosis" with fibroblasts; and the presence of either fibrin or materials very similar to fibrin.

Speculation as to explanations for the heavy periodontal ligament, muscular (temporalis), and periosteal deposits of collagen may run in several directions. It seems probable that such a distribution is favored, first of all, by the stress placed upon these tissues. Increases in connective tissue are, of necessity, initiated from sites where prominent connective tissue already exists. These are the sites where the greatest amounts of collagen are laid down during lathyrism, at least until the process becomes so extensive that the original structure is totally obliterated.

Acknowledging this undoubtedly important factor, there nevertheless exists the peculiarity that the large, acellular hyaline areas of the periodontal ligament are more prominent than the areas immediately surrounding arterioles during lathyrism. Furthermore they do not occur to the same degree perivascularly, as at the locations distant from these vessels, such as in the periodontal ligament proper. Thus there appears to be a superabundance of collagen in proportion to fibroblasts in the periodontal ligament. The natural implication in such a situation is that some material favoring collagen formation is transported by the blood and has priority of usage by elements closest to the afferent circulation. If we fit this postulate to the scheme of connective tissue formation proposed by Altshuler and Angevine,[17] the minimal to moderate perivascular collagen deposits in lathyrism might be augmented by excessive supply of proteins to combine with the acid mucopolysaccharides provided by the fibroblasts.

It seems pertinent to remark on the similarity between the perivascular collars of connective tissue in the spleen in lupus erythematosus disseminata and the nodes in Hodgkin's disease, recalling the proposal of Ehrich[19] that a better term for the "collagen diseases" might be "dysgamma-globulinemias."

The evidence suggests that deposits of the type occurring in lathyrism may represent the sclerosis without fibroplasia described by Rossie[22] as the end result of serous inflammation. Against this concept is the fact that in this study it was not possible definitely to demonstrate ground substance as a matrix for the deposition of collagen in the subendothelial sites. The presence of large fibroblasts surrounding the deposits in most instances suggests that these cells contributed in some manner possibly analogous to their role in amyloid formation.

It is of interest here to recall Teilum's report[27, 28] on what he referred to as "hyalinosis" or "paramyloidosis" in Boeck's sarcoid, lupus erythematosus disseminata, and sulfonamide hypersensitivity. There is little doubt that the perivascular hyaline changes to which he referred may be of a nature very similar to the changes under discussion here in lathyrism. It seems that while the hyaline nature of some of the deposits may well be related to hyperglobulinemia and that their formation may involve mechanisms

similar to those of amyloid formation, nevertheless they react histochemically like collagen, not like amyloid.

The fibrinoid-like material in the lesions of lathyrism is of special interest, since one rarely has the opportunity to study such material unassociated with extremely large amounts of pre-existing connective tissues. Because the fibrinoid-like material characteristically occurred adjacent to collections of proliferating fibroblasts, there also seems justification to wonder whether interaction of some metabolite of the fibroblasts with the adjacent tissues may be responsible for the deposition of hyaline material.

It may not be premature to propose that the interrelationships of the lesions described in lathyrism with those of amyloid disease and with those of a number of hyperglobulinemic states as described by Teilum and others[27, 28] suggest that the connective tissue changes in lathyrism may be the result of the superimposition of an immunity phenomenon (hyperglobulinemia) on a local reparative state in the periodontal ligament and periosteum.

SUMMARY AND CONCLUSIONS

All the available histochemical evidence accumulated in the course of this investigation suggests that in experimental lathyrism the homogeneous, translucent, eosinophilic substance deposited, particularly in the periodontal ligament, is altered collagen (paracollagen). Whether the collagen alteration is due to a hypersensitivity to beta-aminopropionitrile, similar to the mechanism responsible for a similar appearance in a number of disorders now considered collectively as collagen diseases, could not be unequivocally demonstrated by this investigation.

The connective tissues in lathyrism are of a nature between typical collagen and hyaline non-fibrous collagen. With the majority of histochemical methods employed, they are non-fibrous collagen. However, with the aldehyde fuchsin stain after peracetic acid oxidation they are shown to contain a significant quantity of a purple noncollagenous fiber. This fiber is resistant to the *Lathyrus* toxin, whereas reticulin and collagen fibers readily undergo alterations.

The histochemical findings provide evidence that the non-fibrous collagenous bodies produced in lathyrism are not composed of amyloid, paramyloid, fibrinoid, fibrin, sclerosis, hyaline scarring, and mucoid degeneration.

Although the pattern of connective tissue changes is rarely, if ever, exactly duplicated in the collagen diseases, it is not to be inferred that the connective tissues in lathyrism are unequivocally unique, being specific only for experimental lathyrism. Taken individually, these same changes are to be found in a medley of conditions.

The possibility still exists that lathyrism represents either an alteration in both collagen and ground substance or the formation of a soluble-type collagen.

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Toxicity Evaluation of Diethylenetriamine

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Interest in the health aspects of high polymers with a potential dental use has increased rapidly in recent years. The toxicology of major resins now in use in dentistry has been reviewed in the past. The toxicity of the epoxy resins has been evaluated, but there is a paucity of information concerning the toxicity of the amine curing agents for these resins in general and specifically the epoxy curing agent diethylenetriamine. Hine *et al.*[1] fed 1 per cent uncured Epon 828 and Epon 562 to rats for 26 consecutive weeks with no obvious difficulty to the test animals. In addition, they fed rats a diet containing 10 per cent cured 828 over a period of 6 weeks, which resulted in no deleterious effects. In contrast, Lea *et al.* found the uncured epoxy resin to be irritating to 48 per cent of the human subjects when applied to the skin.[2]

Little has been presented on the curing agents used for polymerization of the epoxy resins. Previous biologic testing of the curing agents has been restricted primarily to animals. Smith and Carpenter[3] (1944, 1951) placed the toxicity of diethylenetriamine (D.T.A.) and its N (hydroxyethyl) derivative in the same range perorally and percutaneously, the order of magnitude agreeing with the Hine and Kodama classification of "slightly toxic." They found that neither compound caused death when rats were exposed for 8 hours to saturated vapors.

The toxicity of the derivative was slightly less than the parent compound. Ingeberman and Walton[4] (1956) found that modification of diethylenetriamine by hydroxyalkylation or substitution of cyanoethyl on the nitrogen generally decreased the toxicity.

In this study only D.T.A. was used, because the maximal toxicity of the amine curing agent was the primary concern. The purpose was to determine (1) the initial dermal contact response after 24 and 48 hours, in 50 human subjects previously not exposed to D.T.A., and (2) the sensitizing effect of D.T.A. by reapplying it to the initial site 14 days later.

MATERIALS AND METHODS

Initial contact response.—Standard routine patch tests were employed,[5, 6] using Elastoplast bandages, 7.5 cm. long and 2.5 cm. wide, with a central non-adherent gauze patch 2.5 × 2.5 cm. The patches were applied to the medial aspect of the right forearm. Fifty-four subjects were tested by applying one drop of D.T.A. to the test site with a cotton-tipped cylindrical wood stick. After the drop of D.T.A. had touched the skin, it was rubbed with a cotton applicator stick to assure positive tissue contact. The site of application was then covered by an elastoplast bandage. The test site was observed after 24 and 48 hours.

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Sensitization.—The sensitizing effect of the D.T.A. was determined by reapplying the material in the same manner to approximately the same test site after 14 days.

The reactions were read as negative (0), light (1), moderate (*2), and severe (3). Light reaction consists of a faint pinkish discoloration of the skin with slight scaling at late observation. A moderate reaction consists of a dull-red discoloration with edema, slight maceration, and possibly small petechiae. A severe reaction consists of a painful hemorrhagic inflammatory reaction followed by central necrosis and ulceration.

RESULTS

Four subjects with negative reactions were not used, as there was evidence that the elastoplast bandages had been contaminated or loosened. The test sites on 50 subjects were evaluated. It was found that 9 subjects had positive reactions evaluated after 24 hours; of the 9 subjects that had positive reactions, 6 had slight reactions and 3 had moderate reactions.

Forty-eight hours later, there was no increase numerically in reactions, but there was an increase in the severity in some of the 9 subjects with the positive reactions after 24 hours. Two of the subjects had a light reaction, 3 had a moderate reaction, 4 had a severe reaction.

Fourteen days later, the D.T.A. was reapplied to the original test site and evaluated after 24 hours. In the 50 subjects used, there were 8 reactions—6 light and 2 moderate. All the 9 subjects who had had reactions 24 or 48 hours after the initial application had no reaction after reapplication 14 days later.

DISCUSSION

The incidence of reactions after a period of 24 hours in this study (20 per cent) coincides closely with a previous study[7] (16 per cent) by the author. The most interesting aspect of this study was apparent desensitization of the 9 subjects with initial reactions. The desensitization process apparently had no correlation with the severity of the reaction. After 14 days, desensitization was apparent in all subjects with an initial 24-hour reaction, regardless of severity. This desensitization, using T.E.T.A., was reported earlier by Grandjean,[8] although he did not attempt to qualify his statement. In an effort to relate the classification of "slightly toxic" as Hine *et al.* did with D.T.A., I should like to review some of the testing done on monomeric methyl methacrylate.

Diechman,[9] in a series of animal experiments, found that pure monomer of methyl methacrylate has a toxic effect when applied locally to the skin and on the mucous membrane when inhaled. When air mixed with monomer (19 mg. of monomer to 1 liter of air) was inhaled, the test animals died in 2½–5 hours. Spealman *et al.*[10] found, however, that the effect of the monomer is not particularly cumulative. Although intermittently exposed 3 hours per day for 15 days to a concentration as high as 29 mg. of monomer per liter of air, their test animals survived. In 50 per cent of the patch tests to which they subjected the forearms of 50 medical students who had not previously been exposed to monomer of methyl methacrylate, the results were toxic reactions in the form of an erythema, while patch testing with fully polymerized material caused no reactions in the group of 50 test subjects. The observations of Spealman *et al.* were confirmed by Brunel,[11] who found 16 positive reactions in 20 test subjects (80 per cent positive) to patch testing with pure monomer of methyl methacrylate in individuals previously

never exposed to the monomer. Rink[12] is convinced that residual monomer of methyl methacrylate has a strong toxic effect upon contact with human tissues. In summation, diethylenetriamine, one of the most toxic[1] of amine curing agents for epoxy resins, is no more toxic than monomeric methyl methacrylate.

SUMMARY

Diethylenetriamine was applied to the flexor surface of the forearm of 50 human subjects. Nine subjects had positive reactions after 24 hours; of these, 6 had slight and 3 had moderate reactions. Forty-eight hours later there was no increase numerically, but the severity of the reaction had increased.

Fourteen days later D.T.A. was reapplied to the original site. No reaction recurred in any of the 9 original positive subjects.

Diethylenetriamine is no more toxic than monomeric methyl methacrylate.

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The Effect of Calcium and Phosphorus on Fluoride Absorption

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Reports of the effects of calcium and phosphorus on fluoride utilization may be found throughout the literature. Two Indian workers, Wadhwani[1] and Ranganathan,[2] have concluded that the presence of calcium and phosphate in the diet of rats appeared to protect the animals against the toxic effects of fluoride when growth, survival, and the composition and development of bone were the criteria. Lawrenz and Mitchell[3] reached the same conclusions. Jackson and Train[4] reported that fluoride retention was increased in rats placed on a low-calcium diet. Weddle and Muhler[5] obtained similar results in studies comparing fluoride retention in rats given calcium-supplemented or unsupplemented fluoride drinking water. Some information as to the means by which calcium reduces fluoride retention is furnished by the data of Krylova and Gnoevaya,[6] who reported that the feces fluoride:urine fluoride ratio increased in rats given 0.5 gm. $CaCO_3$ daily. This suggests that the mode of action of calcium and other similarly acting inorganic ions is an inhibiting of the absorption of the fluoride ion. This study was designed to study the effect of calcium and phosphate on fluoride retention when introduced into the gastrointestinal tract simultaneously with, or at selected times following, fluoride administration by the same route.

EXPERIMENTAL METHODS

Seventy-two weanling male rats of the Sprague-Dawley strain were divided by weight into nine groups. Group I served as control and received no treatment. The remainder of the experimental groups received 2 mg. fluoride daily by stomach tube. Groups III, IV, and V were given 2 mg. calcium by stomach tube 15, 30, and 60 minutes, respectively, after fluoride ingestion. Groups VI and VII were given 2 mg. phosphate 15 and 60 minutes, respectively, after fluoride ingestion. Group VIII received 2 mg. calcium and 2 mg. phosphate simultaneously with fluoride administration, and Group IX received calcium and phosphate 15 minutes after fluoride ingestion. In order to minimize interference by ingested food, the experimental animals were intubated following a fasting period of approximately 6 hours, and food was not then

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supplied for an additional 2 hours. Low-fluoride distilled drinking water ($F = 0.01$ ppm) was available at all times.

Analytical grade reagents and redistilled, fluoride-free water were used to prepare the solutions, which were stored in polyethylene bottles. Calcium was furnished as calcium chloride; phosphate as sodium dihydrogen phosphate; and fluoride as sodium fluoride. Stomach intubation was accomplished with a size 8 soft-rubber catheter attached to a 1-ml. tuberculin syringe. After 8 days, the animals were sacrificed, and the carcasses were analyzed for fluoride, using a modification of the Smith and Gardner procedures.[7]

DATA AND DISCUSSION

The results of carcass analyses are presented in Table 1. The net per cent retention was calculated on the basis of the fluoride given experimentally, after discounting the

TABLE 1

RESULTS OF WHOLE-CARCASS ANALYSES OF RATS GIVEN FLUORIDE, CALCIUM, OR PHOSPHORUS UNDER VARIOUS CONDITIONS OF INGESTION

GROUP	DAILY TREATMENT	F IN CARCASS		NET PER CENT RETENTION
		μg.	ppm	
I	Control	904 ± 125*	214 ± 21*	.....
II 2 mg. F^-	.....	9,769 ± 468	2,905 ± 262	54.8
III 2 mg. F^-	2 mg. Ca^{++} after 15 min.	8,545 ± 492	2,516 ± 144	47.3
IV 2 mg. F^-	2 mg. Ca^{++} after 30 min.	8,589 ± 409	2,388 ± 158	47.5
V 2 mg. F^-	2 mg. Ca^{++} after 60 min.	8,269 ± 325	2,401 ± 97	45.5
VI 2 mg. F^-	2 mg. $PO_4^{\equiv}$ after 15 min.	11,078 ± 301	2,839 ± 94	67.8
VII 2 mg. F^-	2 mg. $PO_4^{\equiv}$ after 60 min.	10,351 ± 406	3,081 ± 172	58.5
VIII 2 mg. F^-	2 mg. Ca^{++}, 2 mg. $PO_4^{\equiv}$ (one solution)	7,209 ± 325	2,343 ± 168	38.9
IX 2 mg. F^-	2 mg. Ca^{++}, 2 mg. $PO_4^{\equiv}$ after 15 min.	8,931 ± 434	2,440 ± 96	49.6

* Standard deviation.

carcass fluoride derived from traces in diet and other sources. This fluoride is represented by the carcass fluoride values of the control group. The effect of calcium at 15, 30, and 60 minutes was to reduce the absorption of fluoride appreciably, since the retention of fluoride in the whole carcass was reduced from 54.8 per cent in animals given fluoride alone to 47.3, 47.5, and 45.5 per cent in the animals receiving calcium at 15, 30, and 60 minutes, respectively. There does not appear to be any correlation between the reduction in absorption and the time elapsed between fluoride and calcium ingestion, since there was no significant difference in the retention of fluoride among any of these groups.

These data are interesting in relation to the way in which calcium may act in affecting fluoride utilization. For instance, if the mechanism of action of calcium is the formation of the slightly soluble calcium fluoride, thereby reducing absorbability, this effect would be more evident, the sooner calcium is introduced. In this study calcium administered 1 hour after fluoride ingestion influenced fluoride absorption more than when given 15 minutes following fluoride administration.

The effect of phosphate was to increase the absorption of fluoride, as reflected in the higher proportion retained in these animals when compared with animals receiving fluoride alone. About 9 per cent more fluoride was retained when phosphate was given 15 minutes after fluoride ingestion than when administered after 60 minutes. This phenomenon of increased fluoride absorption in the presence of phosphate may be due to the binding by phosphate of calcium in the gastrointestinal tract, which would otherwise interfere with fluoride absorption. The over-all effect is to allow more absorption of fluoride by removal of the interfering effect of calcium. The increased absorption of fluoride in the presence of phosphorus as acid phosphate cannot be ascribed to a change in the pH of the gastric contents, since (*a*) while a lowering in pH would seem to facilitate fluoride absorption, the added unbuffered phosphate solution had a pH of approximately 4.0, considerably less acidic than gastric juice, and (*b*) the buffering capacity of the small amount of phosphate used would be quite small.

The group receiving calcium, phosphate, and fluoride in one solution retained less fluoride than any other group studied. When calcium and phosphate were given 15 minutes after fluoride administration, however, in Group IX, there was a significant increase in the amount of fluoride retained when compared with Group VIII. Expressed in another way, a significant amount of fluoride ($p = <0.01$, based on a computation of t) had been absorbed in the 15-minute interval. The inhibition of fluoride absorption by calcium plus phosphate was completely contrary to the increased retention resulting from the administration of phosphate alone. The markedly reduced effect of calcium plus phosphate after 15 minutes, compared with that obtained with simultaneous ingestion of calcium, phosphate, and fluoride, suggests that fluoride had been absorbed rapidly and was not available in the gastrointestinal tract after 15 minutes in order to be acted upon by calcium and phosphate introduced at that time.

SUMMARY

When weanling rats were given 2 mg. calcium at 15, 30, and 60 minutes following the administartion of 2 mg. fluoride, a significant ($p = <0.01$) reduction in fluoride retention resulted, when compared with animals receiving fluoride alone. The degree of reduction in fluoride absorption, however, was not time-dependent.

The effect of phosphate given 15 and 60 minutes after fluoride ingestion was a significant ($p = <0.01$) increase in fluoride retention. The influence of phosphate was less pronounced when given after the longer time interval, possibly because of the rapid absorption of fluoride which had taken place before phosphate administration.

When calcium and phosphate were given concurrently with fluoride, a reduction in absorption was obtained amounting to nearly 16 per cent of the fluoride given. The effect of calcium plus phosphate 15 minutes after fluoride ingestion was significantly less, suggesting that most of the fluoride had been absorbed during that time and was not available for interaction with the added calcium and phosphate. Interference with absorption may be the principal means by which dietary calcium and phosphorus interfere with fluoride utilization.

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Studies in the Design and Analysis of Dental Experiments

I. *The Importance of Equal Sample Sizes in Experiments*

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INTRODUCTION

A frequent type of experiment is that in which two treatments are applied to a given group of individuals so that each individual is represented under both treatments. The individual often has several observations under the first treatment and likewise under the second. Consequently, the data for each individual can be thought of as forming a little experiment for assessing the effect of treatment for that individual. Usually, the object of the total experiment on the group of individuals is to estimate the difference between the two treatments for the group and test its significance from zero. Our interest is usually not confined to the individuals being utilized in the experiment but rather extends to the universe of individuals of which our group is a representative sample. The general pattern of this type of data conforms to what is called the "mixed model" in analysis-of-variance terminology.

The analysis and interpretation of such data, i.e., "mixed model," are not difficult if each individual is represented only once for each treatment or if the number of readings in each cell, i.e., individual-treatment combination, is constant. However, if the number of readings in each cell is not constant, e.g., the ith individual has n_{1i} readings under treatment 1 and n_{2i} under treatment 2 and these numbers are unequal and may also differ from the corresponding numbers for the jth individual, then there is no very satisfactory method of analyzing the data *unless* one may assume that the treatment effect does not differ from one individual to the next. In this latter case, one says that there is no "individual-preparation interaction."

Most of the classic methods[1] for handling data of this type refer to cases in which there is no interaction or in which the interest is not in a universe of individuals but rather in the particular group of individuals used (the so-called "fixed model"). In the biological field an individual-preparation interaction is generally found to exist. The

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analysis of such data for unequal replication in the cell has not been developed satisfactorily. We shall discuss various methods by which such data might be analyzed.

DESCRIPTION OF THE EXPERIMENT

In the development of a new adhesive vehicle for obtaining long contact with oral mucous membranes, different formulations or preparations were studied. After preliminary screening, two preparations (X and Y) were applied according to a prearranged random scheme to specific areas of the oral cavity of nine dental students at Columbia University School of Dental and Oral Surgery.[2] Repeated applications were made to the same sites, and each student determined, to the nearest 5 minutes, the duration of contact of the preparation with the tissue in that area. It was not disclosed to the subjects which preparations were being applied. The conduct of the experiment was such that, for various reasons, the number of applications was not the same for each preparation, or for each student. The observations for the alveolar mucosa on the labial of the upper right canine have been selected as illustrative data for this paper, and appear in Table 1.

METHODS OF ANALYSIS

I. *Data trimmed down to equal number of readings in each cell.*—If the readings are given in chronological sequence, the later readings may be eliminated so as to end up with an equal number in each cell. This number will, of course, depend upon the minimum number which any of the original cells has. If the readings are not given in chronological order, the excess readings are eliminated according to some random method, e.g., by random numbers. By this trimming process we end up with a typical two-way analysis of variance with an equal number of readings per cell.[3] The preparation difference may then be estimated and its significance tested by one of several equivalent methods.

1. Replace the readings in each cell by an average and perform one of two equivalent tests (Table 2): (*a*) *t*-test on the preparation difference—this test is quite satisfactory, since our main interest is in the average preparation difference. (*b*) A two-way analysis of variance on the single average reading per cell. It may be noted that the residual mean square is half the variance of the preparation difference calculated in part *a* (s_Δ^2) and is the appropriate denominator for testing the significance of the between-preparation mean square. The size of the residual mean square will reflect any individual-preparation interaction that occurs. Nevertheless, the residual mean square is the appropriate error term for testing between-preparations in the mixed model.

This two-way analysis of variance also gives us the variation between individuals. However, we really do not need this, since we are initially prepared to assume that individuals are at different levels. This is precisely the reason that we insist on using the same individuals in both preparations. We note that the between-individuals mean square is much larger than the residual mean square from Table 2.

The replacement of the readings in each cell by their average, however, does not utilize all the information that is present in the trimmed-down data. By utilizing all the data, it is possible to obtain an estimate of how much an individual varies within himself on the same preparation. Consequently, we would be in a position to test the reality of an individual-preparation interaction.

2. To overcome the inadequacies of the analyses described above in part 1, *a* and *b*, the analysis of variance on the trimmed-down data may be performed directly with the individual measurements (Table 3). The total sum of squares is first subdivided into between cells and within cells. The between-cells sum of squares is then subdivided into between-preparations, between-individuals, and residual sums of squares.

The between-preparations mean square is tested for significance by dividing by the residual mean square. The resulting F is precisely the F *under* 1, *b*, and the square of the t under 1, *a*. The residual mean square can be compared with the within-cells mean

TABLE 1

DURATION OF CONTACT OF TWO ADHESIVE PREPARATIONS (X AND Y) TO ALVEOLAR MUCOSA IN UPPER RIGHT CANINE AREA OF NINE INDIVIDUALS

INDIVIDUAL	DURATION (MIN.)		INDIVIDUAL	DURATION (MIN.)		INDIVIDUAL	DURATION (MIN.)	
	X	Y		X	Y		X	Y
S.S.	165	330	T.S.	100	175	S.C.	60	150
	110	180		155	115		60	90
	205	180		95	85		75	120
	145	240		135	140		45	90
	190	360		70	120		135	75
	125	300		85	260		50	90
		290		105	220		120	70
		265		90	50		105	105
				100	80		45	75
P.T.	85	60		90	80		135	135
	85	100			45			135
	45	30			85			125
	70	90			110			110
	165	120			60			110
	60				85			120
	80				90			60
					70			140
S.R.	70	95			75			105
	125	105						140
	75	120	K.S.	90	90			140
	135	140		90	75			
	135	145		65	60	H.B.	130	260
	130	145		105	120		120	120
	125	140		90	145		510	225
	130	140		120	90		150	120
	135	140		120	135		75	180
	145	140		120	135		120	150
	145	140		120	120		145	135
	130	125		120	120		90	145
		120		120	120			155
		125		100				
		115				R.S.	225	270
			D.S.	50	120		195	180
				35	170		260	225
				120	150		180	260
				75	135		190	250
							195	250
							195	250
							195	205
							225	
							75	
							180	

TABLE 2

t-TEST AND ANALYSIS OF VARIANCE ON AVERAGES OF DATA TRIMMED TO EQUALITY WITH 4 READINGS PER CELL

INDIVIDUAL	MEAN OF 4 CELL READINGS		DIFF. BETWEEN MEANS (Δ) Y−X	INDIVIDUAL TOTAL OF 2 MEANS X+Y
	X	Y		
S.S.	156.25	232.50	+ 76.25	388.75
P.T.	71.25	70.00	− 1.25	141.25
S.R.	101.25	115.00	+ 13.75	216.25
T.S.	121.25	128.75	+ 7.50	250.00
K.S.	87.50	86.25	− 1.25	173.75
D.S.	70.00	143.75	+ 73.75	213.75
S.C.	60.00	112.50	+ 52.50	172.50
H.B.	227.50	181.25	− 46.25	408.75
R.S.	215.00	233.75	+ 18.75	448.75
Total of 9 cell means	1,110.00	1,303.75	+193.75	2,413.75 = Grand total of 18 cell means

t-Test

$$s^2_\Delta = \frac{N\Sigma\Delta^2 - (\Sigma\Delta)^2}{N(N-1)} = \frac{9(16{,}748.4375) - (37{,}539.0625)}{72} = 1{,}572.1788,$$

$$s_{\bar{\Delta}} = \sqrt{\frac{s^2_\Delta}{N}} = \sqrt{\frac{1{,}572.1788}{9}} = 13.22,$$

$$t = \frac{\bar{\Delta}}{s_{\bar{\Delta}}} = \frac{21.53}{13.22} = 1.63 \text{ with 8 degrees of freedom } (d).$$

Analysis of Variance

$$\text{Correction term (CT)} = \frac{(\text{grand total})^2}{(9)(2)} = \frac{(2{,}413.75)^2}{18} = 323{,}677.1701;$$

$$\text{Between-preparations SS} = \frac{(\text{total of 9 cell means for X})^2 + (\ldots \text{for Y})^2}{9} - \text{CT}$$

$$= \frac{[(1{,}110.00)^2 + (1{,}303.75)^2]}{9} - \text{CT};$$

$$\text{Between-individuals SS} = \frac{(\text{individual total of 2 cell means})^2}{2} - \text{CT}$$

$$= \frac{[(388.75)^2 + (141.25)^2 + \ldots + (448.75)^2]}{2} - \text{CT};$$

$$\text{Total SS} = \text{Sum of (each cell mean)}^2 - \text{CT}$$

$$= [(156.25)^2 + (71.25)^2 + \ldots + (233.75)^2] - \text{CT}$$

Source of Variation	*d*	Sum of Squares (SS)	Mean Square (MS)
Between preparations	1	2,085.5035	2,085.5035
Between individuals	8	53,537.6736	6,692.2092
Residual*	8	6,288.7153	786.0894
Total	17	61,911.8924	.....

* SS obtained by subtraction.

$$t^2 = F = \frac{\text{Between-preparations MS}}{\text{Residual MS}} = \frac{2{,}085.5035}{786.0894} = 2.65;\ d_1 = 1,\ d_2 = 8.$$

TABLE 3

ANALYSIS OF VARIANCE TRIMMED TO EQUALITY, WITH 4 READINGS PER CELL

INDIVIDUAL	SUM OF 4 CELL READINGS		INDIVIDUAL TOTALS OF 8 CELL READINGS X+Y
	X	Y	
S.S.	625	930	1,555
P.T.	285	280	565
S.R.	405	460	865
T.S.	485	515	1,000
K.S.	350	345	695
D.S.	280	575	855
S,C.	240	450	690
H.B.	910	725	1,635
R.S.	860	935	1,795
Total of 36 cell readings	4 440	5,215	9,655 = Grand total of 72 cell readings

$$CT = \frac{(\text{grand total of 72 cell readings})^2}{72} = \frac{(9,655)^2}{72} = 1,294,708.6805;$$

$$\text{Between-preparations SS} = \frac{(\text{X total})^2 + (\text{Y total})^2}{36} - CT$$

$$= \frac{[(4,440)^2 + (5,215)^2]}{36} - CT;$$

$$\text{Between-individuals SS} = \frac{\text{Sum of (individual total)}^2}{8} - CT$$

$$= \frac{[(1,555)^2 + (565)^2 + \ldots + (1,795)^2]}{8} - CT;$$

$$\text{Between-cells SS} = \frac{\text{Sum of (cell sum)}^2}{4} - CT$$

$$= \frac{[(625)^2 + (285)^2 + \ldots + (935)^2]}{4} - CT;$$

$$\text{Total SS} = \text{Sum of 72 (cell reading)}^2 - CT$$

$$= [(165)^2 + (110)^2 + \ldots + (260)^2] - CT.$$

Source of Variation	d	Sum of Squares (SS)	Mean Square (MS)
Between preparations	1	8,342.014	8,342.014
Between individuals	8	214,150.695	26,768.837
Residual*	8	25,154.861	3,144.358
Between cells*	17	247,647.570	.....
Within cells	54	177,668.750	3,290.162
Total	71	425,316.320	.....

* SS obtained by subtraction.

$$F = \frac{\text{Between-preparations MS}}{\text{Residual MS}} = \frac{8,342.014}{3,144.358} = 2.65;\ d_1 = 1,\ d_2 = 8;$$

$$F = \frac{\text{Residual MS}}{\text{Within-cells MS}} = \frac{3,144.358}{3,290.162} = 0.95;\ d_1 = 8,\ d_2 = 54.$$

square to see whether there is a significant individual-preparation interaction. In our example, F is approximately 1, indicating a lack of significance. This suggests that, were we to utilize all the data, the interaction, in any event, would not be very large.

The inadequacy of the analysis in terms of the trimmed-down data is the lack of utilization of all the data. There is often very little justification for the unequal replication in the cell presented by the experimenter other than his whim and his lack of appreciation of how an asymmetrical design can produce difficulties in the analysis. Perhaps one way of convincing him of the utility of the symmetrical design is to discard his excess measurements.

II. *Data trimmed down to some type of proportionality.*—When the number of readings per cell is not constant, the analyses appearing in Table 2 are no longer exact. The type of analysis of variance appearing in Table 3 cannot be performed except in the special case where there is some type of proportionality between the number of readings in the various cells such that n_1/n_2 is constant for all individuals. That is, each individual participates in the first preparation to the same relative extent that he does in the second preparation. Thus, for example, if $n_1/n_2 = \frac{1}{2}$, there might be 2 readings for Preparation X and 4 readings for Preparation Y for the first individual; 3 readings for X and 6 for Y for the second individual; 7 for X and 14 for Y for the third; and so on. The type of analysis of variance illustrated in Table 3 may then be performed with but minor complications.

The inequalities of the numbers of measurements per cell in the present data do not give us proportionality. If we eliminate the few extra readings that the ith individual has with one preparation or the other, we end up with a rather simple type of proportionality, such that $n_{1i} = n_{2i}$. The analysis of the data is then fairly direct, being complicated only by the fact that the number of readings per cell is not constant and the number per individual is not constant. In the particular proportionality we have here, the number per preparation is, however, constant.

The first step in the analysis is to compute the total and the between-cells sums of squares (Table 4). The latter is obtained differently from that shown in Table 3, since each squared cell sum has to be divided by the number of readings in the cell instead of delaying the division until the end. The within-cells sum of squares is obtained by subtraction. The between-cells sum of squares is decomposed into between-individuals, between-preparations, and residual sums of squares. The decomposition differs, in this case, from that in Table 3 because each individual is based on a different number of readings, so that the squares of the individual sums have to be divided by different numbers. The residual sum of squares is obtained by subtraction, since the addition theorem of the sum of squares holds in this case of proportionality just as it did in the case of equality.

By using as many of the original data as is consistent with proportionality—namely, 144 readings—we find that the residual mean square approaches significance at the 5 per cent level when compared with the within-cells mean square. The fact that there may well be a real individual-preparation interaction, as would indeed have been conjectured in view of the fact that different individuals are concerned, means that there may be no correct error term present in the analysis of variance for the test of the significance of the between-preparations mean square. The comparison of the between-preparations mean square with the residual mean square, as was done in Table 3, is

TABLE 4

ANALYSIS OF VARIANCE OF DATA TRIMMED TO PROPORTIONALITY BY MAKING NUMBER OF X READINGS FOR EACH INDIVIDUAL EQUAL TO NUMBER OF Y READINGS

INDIVIDUAL	NO. READINGS PER INDIVIDUAL PER CELL (n_i)	SUM OF CELL READINGS		INDIVIDUAL SUM OF $2n_i$ CELL READINGS
		X	Y	
S.S.	6	940	1,590	2,530
P.T.	5	450	400	850
S.R.	11	1,335	1,435	2,770
T.S.	10	1,025	1,325	2,350
K.S.	11	1,160	1,210	2,370
D.S.	4	280	575	855
S.C.	10	830	1,000	1,830
H.B.	8	1,340	1,335	2,675
R.S.	7	1,440	1,640	3,080
Total of 72 readings	72	8,800	10,510	19,310 = Grand total of 144 readings

$$CT = \frac{(\text{Grand total of 144 cell readings})^2}{144} = \frac{(19{,}310)^2}{144} = 2{,}589{,}417.3611;$$

$$\text{Between-preparations SS} = \frac{(\text{X total})^2 + (\text{Y total})^2}{72} - CT$$

$$= \frac{[(8{,}800)^2 + (10{,}510)^2]}{72} - CT;$$

$$\text{Between-individuals SS} = \text{Sum of } \left[\frac{(\text{individual total})^2}{2n_i}\right] - CT$$

$$= \left[\frac{(2{,}530)^2}{12} + \frac{(850)^2}{10} + \ldots + \frac{(3{,}080)^2}{14}\right] - CT;$$

$$\text{Between-cells SS} = \text{Sum of } \left[\frac{(\text{cell sum})^2}{n_i}\right] - CT$$

$$= \left[\frac{(940)^2}{6} + \frac{(1{,}590)^2}{6} + \ldots + \frac{(1{,}640)^2}{7}\right] - CT;$$

$$\text{Total SS} = \text{Sum of } 144\,(\text{cell reading})^2 - CT$$

$$= [(165)^2 + (110)^2 + \ldots + (105)^2] - CT.$$

Source of Variation	d	Sum of Squares (SS)	Mean Square (MS)
Between preparations	1	20,306.2500	20,306.2500
Between individuals	8	280,097.4778	35,012.1847
Residual*	8	35,402.0954	4,425.2619
Between cells	17	335,805.8232	.....
Within cells*	126	308,326.8157	2,447.0382
Total	143	644,132.6389	.....

* SS obtained by subtraction.

$$F = \frac{\text{Between-preparations MS}}{\text{Residual MS}} = \frac{20{,}306.2500}{4{,}425.2619} = 4.59;\ d_1 = 1,\ d_2 = 8;$$

$$F = \frac{\text{Residual MS}}{\text{Within-cells MS}} = \frac{4{,}425.2619}{2{,}447.0382} = 1.81;\ d_1 = 8,\ d_2 = 126.$$

TABLE 5

t-TEST OF AVERAGES OF ALL DATA FOR EACH INDIVIDUAL WITH EACH PREPARATION, AND ANALYSIS OF VARIANCE (WEIGHTED) OF ALL DATA

INDIVIDUAL	PREPARATION X			PREPARATION Y			DIFFERENCE BETWEEN AVERAGES Y−X (Δ)	SUM OF $n_{xi}+n_{yi}$ CELL READINGS	WEIGHT (w_i) $(1/n_{xi}+1/n_{yi})$
	No. Cell Readings per Ind. n_{xi}	Sum of Cell Readings	Average Cell Reading X	No. Cell Readings per Ind. n_{yi}	Sum of Cell Readings	Average Cell Reading Y			
S.S.	6	940	156.667	8	2,145	268.125	+111.458	3,085	3.4281
P.T.	7	590	84.286	5	400	80.000	− 4.286	990	2.9163
S.R.	11	1,335	121.364	14	1,795	128.214	+ 6.850	3,130	6.1614
T.S.	10	1,025	102.500	18	1,945	108.056	+ 5.556	2,970	6.4267
K.S.	12	1,260	105.000	11	1,210	110.000	+ 5.000	2,470	5.7405
D.S.	4	280	70.000	4	575	143.750	+ 73.750	855	2.0000
S.C.	10	830	83.000	20	2,185	109.250	+ 26.250	3,015	6.6666
H.B.	8	1,340	167.500	9	1,490	165.556	− 1.944	2,830	4.2354
R.S.	10	1,920	192.000	7	1,640	234.286	+ 42.286	3,560	4.1169
Total	78	.....	1,082.3170	96	.....	1,347.2370	+264.920	22,905	41.6919

t-Test of Averages

$$s^2_\Delta = \frac{N\Sigma\Delta^2 - (\Sigma\Delta)^2}{N(N-1)}$$

$$= \frac{9[(111.458)^2 + (-4.286)^2 + \ldots + (42.286)^2] - (264.920)^2}{72} = 1{,}583.2487;$$

$$s_{\bar{\Delta}} = \sqrt{\frac{s^2_\Delta}{N}} = \sqrt{\frac{1{,}583.2487}{9}} = 13.263;$$

$$t = \frac{\bar{\Delta}}{s_{\bar{\Delta}}} = \frac{264.920/9}{13.263} = 2.219,\ d = 8.$$

Analysis of Variance:

$$\text{CT} = \frac{(\text{grand total of 174 readings})^2}{174} = \frac{(22{,}905)^2}{174} = 3{,}015{,}166.8103;$$

$$\text{Between-individuals SS} = \text{Sum of}\left[\frac{(\text{individual total})^2}{n_{xi}+n_{yi}}\right] - \text{CT}$$

$$= \left[\frac{(3{,}085)^2}{14} + \frac{(990)^2}{12} + \ldots + \frac{(3{,}560)^2}{17}\right] - \text{CT};$$

$$\text{Between-cells SS} = \text{Sum of}\left[\frac{(\text{cell sum})^2}{n_{xi} \text{ or } n_{yi}}\right] - \text{CT}$$

$$= \left[\frac{(940)^2}{6} + \frac{(2{,}145)^2}{8} + \ldots + \frac{(1{,}640)^2}{7}\right] - \text{CT};$$

$$\text{Total SS} = \text{Sum of 174 (cell reading)}^2 - \text{CT}$$

$$= [(165)^2 + (110)^2 + \ldots + (205)^2] - \text{CT};$$

$$\text{Weighted average difference} = \bar{\Delta}_w = \frac{\text{Sum of } w_i\Delta_i}{\text{total weight}}$$

$$= \frac{(3.4281)(111.458) + \ldots + (4.1169)(42.286)}{41.6919};$$

$$\text{Adjusted between-preparations SS} = \frac{(\text{sum of } w_i\Delta_i)^2}{\text{total weight}} = \frac{(964.5684)^2}{41.6919}.$$

Source of Variation	d	Sum of Squares (SS)	Mean Square (MS)
Adjusted between preparations..	1	22,315.898	22,315.898
Between individuals.....	8	329,477.912	41,184.739
Residual*.....	8	43,812.245	5,476.531
Between cells.....	17	395,606.055	.....
Within cells*.....	156	349,702.135	2,241.6804
Total.....	173	745,308.190	.....

* SS obtained by subtraction.

$$F = \frac{\text{Adjusted between-preparations MS}}{\text{Residual MS}} = \frac{22{,}315.898}{5{,}476.531} = 4.07;\ d_1 = 1,\ d_2 = 8;$$

$$F = \frac{\text{Residual MS}}{\text{Within-cells MS}} = \frac{5{,}476.531}{2{,}241.680} = 2.44;\ d_1 = 8,\ d_2 = 156.$$

not quite correct in the case of the mixed model, except with certain special types of proportionality. In the present example, the true interaction could not be very large at any rate, so that the test suggested is not too bad. The difference between preparations is still not significant, although it corresponds to a lower probability than in the case of equality.

III. *Utilizing all the data.*—All the data may be utilized by replacing the number of readings in each cell by their mean and performing a t-test on the average preparation difference, or the equivalent analysis of variance with one reading per cell (Table 5). While this is certainly not exact because each mean is given the same weight, it does approach correctness as the real interaction becomes large. In this method we do not have a test for the significance of the interaction.

2. An analysis of variance may be performed by utilizing all the data, but it is more difficult than that illustrated in Table 4 because the between-cells sum of squares cannot be subdivided so easily into its three components, since the addition theorem fails when there is non-proportionality. The total sum of squares is divisible into between-cells and within-cells sums of squares. Again, in the computation of between-cells sum of squares, each squared cell sum must be divided by its appropriate number of readings, as in Table 4. The between-individuals sum of squares is computed in the usual manner, as in Table 4, paying attention to the unequal number of readings per individual. In order to compute between-preparations sum of squares, however, weights have to be used which reflect the inequalities of the number of readings per cell. The weight, w_i, for the preparation difference, Δ_i for the ith individual, is proportional to the reciprocal of its variance,

$$w_i = \frac{1}{(1/n_{1i}) + (1/n_{2i})}.$$

The weighted average preparation difference is then computed as

$$\bar{\Delta}_w = \frac{\Sigma (w_i \Delta_i)}{\Sigma (w_i)} = 23.14 .$$

It will be noted that $\bar{\Delta}_w$ does not differ greatly in magnitude from an unweighted average of the Δ's ($\bar{\Delta} = 28.34$). The adjusted between-preparations sum of squares is computed as

$$\frac{[\Sigma (w_i \Delta_i)]^2}{\Sigma (w_i)} .$$

The residual sum of squares is now computed as follows: Bet. Cells SS — Bet. Individuals SS — Adj. Bet. Preparations SS.

Actually, in the analysis of variance of the data trimmed to equality or proportionality, the same weighting procedure as that just illustrated was followed. However, because of the simpler relationship of the number of readings in the various cells, the arithmetic became simplified so that the weighting was not apparent.

The individual-preparation interaction is tested for significance by comparing the residual mean square with the within-cells mean square. Since the interaction is significant at the 5 per cent level, the comparison of the adjusted between-preparations mean square with the residual mean square is not quite correct. Since the interaction does not appear to be large, the test suggested appears to be reasonable. Some modification of the weighting procedure which would take into account not only the unequal number of readings but also the true interaction variance might be desirable, however.

If the interaction were judged to be large, the analysis mentioned in Section III, 1, working with the unweighted Δ's, is preferable. If there were no interaction, the comparison of between-preparations mean square with residual mean square would be exactly correct. In such a case, however, one could use the within-cells mean square with its greater number of degrees of freedom (thereby increasing the power of the test) as the denominator for the F test.

CONCLUSIONS

In the mixed model discussed in this paper, the advantages of having an equal number of readings for each individual for each treatment (i.e., equals readings per cell) are that the arithmetical work is much simpler and that there is an exact significance test for the average difference between preparations when there is an individual-preparation interaction. Proportionality helps by facilitating the calculation of the components in the analysis of variance and in some cases may afford an exact significance test.

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Nutrition and Caries

X. *Observations in Wild Rats*

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The occurrence of dental caries in wild rats is often denied, although its presence was observed long ago.[1] The idea of strong caries resistance of wild rats, however, seems to be based more on prejudice than on observations.

EXPERIMENTAL METHODS

An opportunity to investigate the teeth of about fifty wild rats was offered by the Amsterdam Rat Extermination Service. The heads of the animals were sent in lysol; hence detailed information about the size or sex of the animals or about the age could not be obtained. All rats were brown- to nearly black-haired (*Mus norvegicus*). The find-spots varied: most came from shipholds and dockyards, others from sewers and rubbish dumps, and a few had been found in private houses in the "old city" of Amsterdam, including a confectioner's store.

After dissecting, placing in 10 per cent formalin, and examination under a binocular loupe (magnification 15 times), the jaw quadrants were cut longitudinally into sections, according to the techniques of the microscopic caries recording method.[2] This time, four sections of each jaw quadrant were made instead of two.

A more or less arbitrary classification of the rats was made into three groups: (*a*) "old rats," judged by the distinctly visible wear of the molars and the appearance of the skull; (*b*) and (*c*) two groups divided according to the size of the molars. The height as well as the mesiodistal dimensions were taken into account. Group *b* had the higher values and group *c* the lower values for both dimensions.

RESULTS AND DISCUSSION

Two of the 51 rats proved to have macroscopically visible extensive carious lesions. One had been found in a shiphold, the other in a confectioner's store. Both animals seemed to be old specimens. On microscopic evaluation, another 16 animals proved to have one or more carious fissures. Apart from the 2 animals already mentioned with severe cavities, there were 2 others with small enamel defects. All other lesions showed pathological staining with fuchsin only.

Klein[1] reported that 39 of 69 adult wild rats showed dental decay in the lower molars. The remaining 30 were young individuals (small size) and showed no gross dental

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decay. These rats were collected from traps placed about the city dumps. Of course, no direct comparison is possible between Klein's figures and the present ones. This is demonstrated once more by the effect of the classification. Apart from the 2 "old" animals with extensive lesions, there were another 12 animals in this group (*a*); 2 of these 12 showed one small fissure lesion each, without loss of surface continuity. Of the 29 group *b* animals, 5 showed one fissure lesion each, 2 had two lesions each, and 1 other had three lesions. All lesions showed pathological staining with fuchsin only. One animal had a fracture of the distal cusp of the first molar. On the other hand, 6 of the 9 animals in group *c* (small dental elements) proved to have caries; 2 of them already had enamel defects; 5 of these animals with carious lesions had been sent together with 3 animals without lesions on the same occasion. Probably they were from one rat colony, but it is impossible to prove this. Three of these animals had one lesion, 1 had five lesions, another had nine, and the sixth had at least seven, but, as one lower quadrant broke into small pieces on cutting and could not be evaluated, the total number very probably would have been greater. These data indicate the number of fissures in which both enamel and dentino-enamel junction showed attack. There were, however, some fissures which seemed to have enamel attack only.

Approximal lesions were present also. Most of the lesions were of the chalky-white type with some loss of enamel translucency. A few stained with fuchsin, but no cavities occurred. The picture was completely the same as is seen in the Wistar strain laboratory rats.[2] In all three groups one or more lesions occurred in many of the animals.

Accurate records are not really possible because, unfortunately, some jaw parts got lost. The molars of these jaws were so loosely fixed in the bone that a number of them, on cutting, were broken out of the alveolus instead of being cut in the right way. This has never occurred with the preparations of the laboratory rats. Accordingly, it has been observed that in some animals rather large parts of the roots of the molars were visible above the rim of the alveolar bone. This was the case in 6 of the 9 animals of group *c*, and in only 1 of the 29 group *b* animals. The picture seemed to resemble paradentosis, but no work could be carried out to investigate this further.

It was noticed as a peculiarity that a rather high number of third molars in these wild rats had been attacked (in 8 animals one or more M_3's). Contrary to this, our laboratory rats often still have a sound third molar, even when the first and second molars have extensive cavities. On the other hand, it proved to be possible to change this picture by dietary measures: for instance, on giving McClure's[3] cereal diet, an unusually high frequency of third-molar attack was also seen.

The distribution of the lesions in the maxilla and the mandibula seemed to be the same in the wild rats as in the laboratory animals. As long as only few and small lesions are present, there is no predilection for the upper or the lower jaw. With the progress of the attack, the extension of the mandibular lesions goes much faster than in the maxillary molars.

Apart from lesions in the early stages, lamellae were found in some animals. Some, but not all, lamellae stained with fuchsin. In the laboratory rats such lamellae are frequently seen in cases where carious attack of the dentino-enamel junction is evident, with or without severe attack on the enamel, judged by the staining properties with fuchsin. The presence of only red-staining lamellae can very probably be regarded as the first demonstrable sign of carious attack of this type. A staining lamella only is not

a rare finding in the fissures most liable to carious attack in young animals on a cariogenic ration. Such animals will certainly develop extensive lesions in these fissures after a longer experimental period. In histological investigations carried out previously, it has been proved that lamellae and the zone of attack of the dentino-enamel junction are intimately connected with each other, and no line of separation could be found.[4]

All wild animals were found in the neighborhood of food preserved for human consumption. A number of food products to be considered, then, such as rice and corn, certainly are cariogenic for the rat, although wheat is not.[5] Processed cereals[3] might even play a very important role here. Further, milk powder,[6-7] sugar and sugar-containing foods,[8-10] and many preserved and manufactured food products are known to be cariogenic.[11-13] Effects similar to those reported by other authors could also be found in the Institute's Wistar strain rats.[14-18] It would certainly have been of interest to know more details of the wild animals' food, but, unfortunately no conclusive data can be obtained for a sample of wild rats.

SUMMARY AND CONCLUSIONS

Wild rats from shipholds, sewers, etc., developed more or less severe carious lesions. Apart from a possible influence of the type of rat, the kind of food available at the site where it is found might have played a role. Periodontal disease also developed in such animals.

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In Vitro *Culture of Mucosa*

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This investigation was undertaken to determine whether normal growth and maturation patterns of certain mucosae can be reproduced in vitro. Such a study is a prerequisite to investigating the repair of oral mucosa, the growth of tumors of the oral cavity, and the epithelial proliferation of periodontal disease.

In vivo, masticatory mucosa, like skin, is a covering tissue which matures with a keratinized surface.[1] The dental epithelium of the tooth bud does not develop a keratinized surface and proliferates in a budding pattern.[2] Respiratory mucosa is pseudostratified, contains goblet cells, and is ciliated. Like all surface epithelia, it, too, has a covering function.

Skin,[3-7] dental epithelium,[8, 9] and respiratory mucosa[10] have been cultured in vitro. They all show growth and maturation potentials similar to the in vivo development.

This investigation established that masticatory mucosa, dental epithelium, and respiratory mucosa may be successfully cultured as organs.

MATERIALS AND METHODS

Thirty-one embryonic explants were cultured in vitro. Masticatory mucosa, dental epithelium, and respiratory mucosa were dissected from Sherman albino rat fetuses of known age. The masticatory mucosa was taken from the hard palate and the crest of the ridge, the dental epithelium from the tooth bud, and the respiratory mucosa from the floor of the nasal cavity.

The explants were cut into strips of 1×2 mm. and held in Tyrode's solution until four to six tissues could be planted at the same time.

The culture medium was as follows: 20 per cent rat serum, 1 per cent 19-day rat embryo extract, 10 per cent thrombin–phenol red (0.005 per cent) in Tyrode's solution, and 69 per cent heparinized chicken plasma. Each cubic centimeter of the medium contained 100–300 units of bovine thrombin to facilitate clotting.

Two cubic centimeters of the medium were placed in a tissue culture flask. After the medium had coagulated, the tissues were planted on the surface of the clot. Two to 12 hours later, another cubic centimeter of the same medium was applied over the explants.[8]

When the additional medium had clotted, the stoppers were sealed with paraffin. The pH of the medium was 7.4. The cultures were incubated at 37° C.

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The explants were cultured for a maximum of 10 days. They were observed in the flask at 24-hour intervals and photographed. Explants that produced an abundance of outgrowth were subcultured. The outgrowth was discarded from the original explants, which were then replanted in fresh nutrient medium.[8]

Explants were fixed after 2, 4, 7, 8, or 10 days of culture and processed for histologic examination. The serial sections were stained with hematoxylin and eosin.

Table 1 lists the age of the fetus at the time the explants were taken, the tissues cultured, and the number of each tissue cultured.

TABLE 1

Tissue	Age of Fetus (Days)	No. of Explants	Tissue	Age of Fetus (Days)	No. of Explants
Masticatory mucosa (hard palate)	15	9	Tooth bud without outer enamel epithelium	19	4
Masticatory mucosa (hard palate)	19	8	Respiratory mucosa	15	1
Masticatory mucosa (crest of the ridge)	19	4	Respiratory mucosa	19	1
Tooth bud with outer enamel epithelium	19	4			

RESULTS

Masticatory mucosa.—Palatal Mucosa—15-Day Rat Fetus: The epithelium of the masticatory mucosa from the hard palate of a 15-day rat fetus consists of three to four layers of cells. There are no rete pegs, nor is there a granular layer. The surface is not keratinized (Fig. 1).

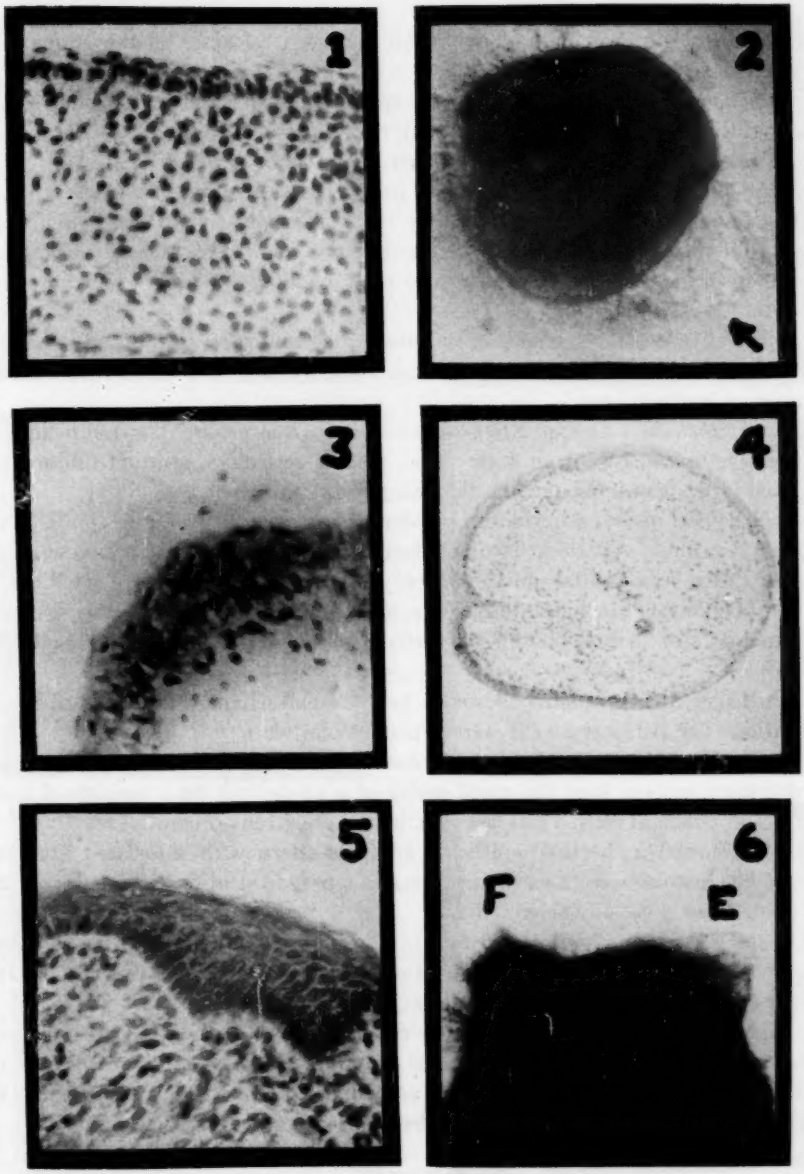
After 1 day of cultivation there was a slight sheetlike growth of epithelium observed in the flask. The growth was irregular. The epithelial outgrowth increased for 4 days of cultivation, at which time slight fibroblastic outgrowth was evident. Lysis of the clot with degeneration of the epithelial outgrowth also occurred after 4 days (Fig. 2). At this stage the explants were subcultured for an additional 4 days. There was very little, if any, outgrowth at the termination of the experiment on the eighth day.

Histologic examination of the explants showed a thin surface layer of keratin 2 days after planting (Fig. 3). Four days of cultivation showed that the tissue had rounded up into a ball of connective tissue covered with a mature, keratinized epithelium (Fig. 4). The epithelium assumed its covering and protective function; it surrounded and covered the exposed submucosa. There was active mitosis in the prickle-cell layer.

Specimens cultivated for 8 days showed an additional advance in development. The epithelium was thicker, with beginning rete-peg formation. The granular layer was present and the surface layer keratinized (Fig. 5). The epithelium appeared similar to sections of palatal epithelium of the 20-day rat fetus.

Palatal Mucosa—19-Day Rat Fetus: The epithelium of 19-day rat fetus masticatory mucosa from the border of the hard palate consists of mature cells with a keratinized surface. Rete pegs are beginning to form.

One day of cultivation produced slight epithelial outgrowth. There was little further evidence of outgrowth throughout the 10 days of cultivation. At the termination of the experiment there was a minimum of epithelial sheetlike and fibroblastic outgrowth



FIGS. 1–6.—**Fig. 1**: Control section: masticatory mucosa from the hard palate of a 15-day rat fetus. Orig. mag. 90×. **Fig. 2**: Explant of masticatory mucosa from the hard palate of a 15-day rat fetus after 4 days of culture. Arrow points to lysis and degeneration. Orig. mag. 35×. **Fig. 3**: Section of masticatory mucosa from the hard palate of a 15-day rat fetus. The explant was cultured 2 days. Orig. mag. 270×. **Fig. 4**: Section of masticatory mucosa from the hard palate of a 15-day rat fetus. The explant was cultured 4 days. Orig. mag. 60×. **Fig. 5**: Section of masticatory mucosa from the hard palate of a 15-day rat fetus. The explant was cultured 8 days. Orig. mag. 90×. **Fig. 6**: Explant of masticatory mucosa from the hard palate of a 19-day rat fetus. The photograph shows the minimal sheetlike epithelial (*E*) and fibroblastic (*F*) outgrowth after 6 days of culture. Orig. mag. 35×.

(Fig. 6). Histologic examination showed an increased thickness of the granular and keratinized layers (Fig. 7).

Ridge Mucosa—19-Day Rat Fetus: The epithelium of the masticatory mucosa from the area of the crest of the ridge of a 19-day rat fetus consists of mature cells. There is begining rete-peg formation, a granular layer, and a keratinized surface.

Explants of the tissue matured the same as did the palatal mucosa. Observed in the flask, they show epithelial outgrowth after 1 day of cultivation. There was no fibroblastic proliferation. From 2 to 6 days of cultivation showed a progressive increase of sheetlike epithelial growth. There was also some budding epithelium and fibroblastic outgrowth.

Histologically, the development of cultured ridge mucosa was the same as palatal mucosa. One explant of masticatory mucosa from the area of the crest of the ridge developed an epithelial pearl.

Dental epithelium.—Dental Epithelium—19-Day Rat Fetus: The tooth bud of a 19-day rat fetus consists of an outer tunic, stellate reticulum, stratum intermedium, ameloblast layer, dental papilla, and the dental sac.[11]

The cultivation of an entire tooth bud showed essentially a budding epithelial outgrowth, as previously reported.[8, 9] In an effort to determine whether budding proliferation arose from outer dental epithelium only, this layer was removed. This left the stellate reticulum, the stratum intermedium, and the ameloblast layers. When cultured, these explants also showed budding epithelial and slight fibroblastic outgrowth (Fig. 8).

Histologically, these explants showed a mass of epithelium with a central area of organization. The peripheral cells were pre-ameloblasts.

Respiratory mucosa.—Respiratory Mucosa—15-Day Rat Fetus: The epithelium of the respiratory mucosa from the nasal cavity of a 15-day rat fetus consists of one to two layers of cuboidal cells. There are no cilia or goblet cells present.

Explants showed a sheetlike epithelial growth pattern with associated fibroblasts (Fig. 9). The histologic sections showed a mature, pseudo-stratified, ciliated, columnar epithelium. There were no goblet cells.

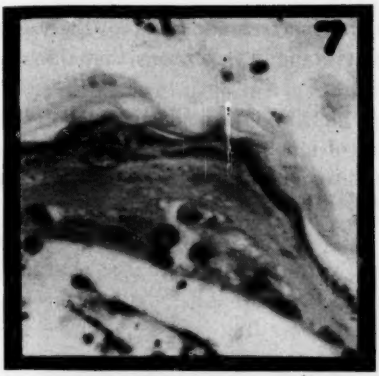
Respiratory Mucosa—19-Day Rat Fetus: The epithelium of the respiratory mucosa from the nasal cavity of a 19-day rat fetus consists of a pseudo-stratified, ciliated, columnar epithelium. Goblet cells are absent.

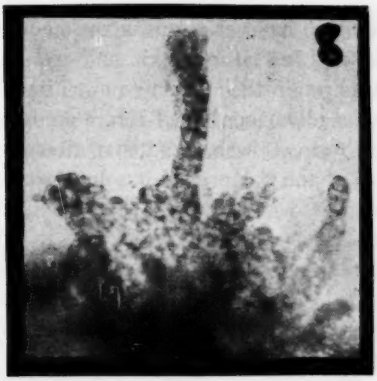
The explant showed evidence of sheetlike epithelial and slight fibroblastic outgrowth. Histologically, this tissue showed a matured respiratory epithelium after 4 days of culture. The epithelium was pseudo-stratified and ciliated. Goblet cells were present (Fig. 10). This stage of development corresponded to 21 days of development in vivo.

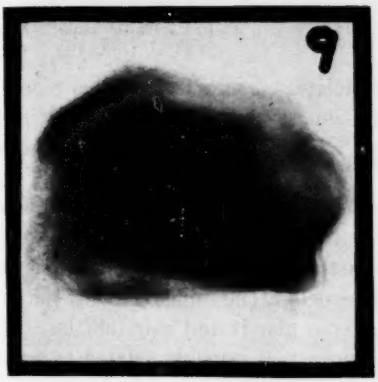
DISCUSSION

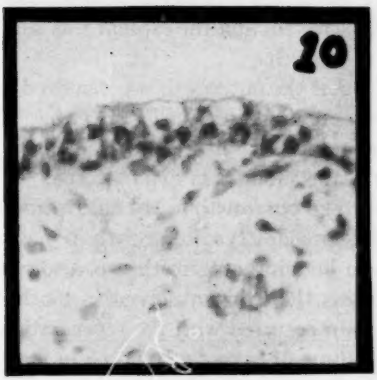
This study was concerned with the maturation of the embryonic tissues in vitro. An established organ-culture technique was used to study growth, differentiation, and maturation.[8]

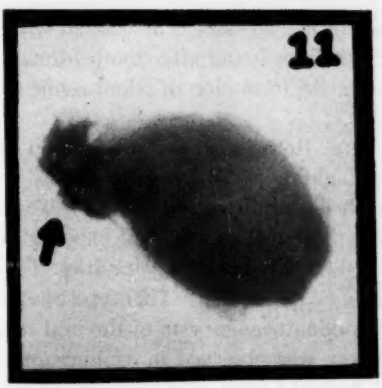
The variable results in skin experiments as well as in this investigation may be attributed to the biologic nutritional medium. Only a synthetic medium can provide a constant nutritional environment. As yet, no synthetic medium has been established for organ culture. In general, the results of this study are comparable to skin investigations,

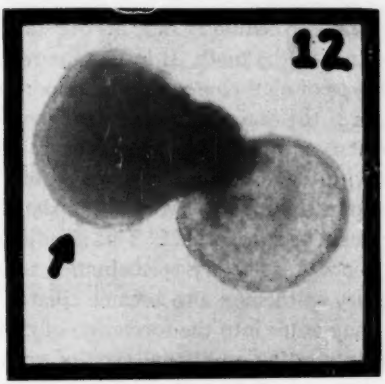












FIGS. 7–12.—Fig. 7: Section of masticatory mucosa from the hard palate of a 19-day rat fetus. The explant was cultured 10 days. Orig. mag. 360×. Fig. 8: Explant of a tooth bud from a 19-day rat fetus after 4 days of culture. The outer tunic was removed during dissection. Orig. mag. 100×. Fig. 9: Explant of respiratory mucosa from a 15-day rat fetus after 1 day of culture. Orig. mag. 7×. Fig. 10: Section of respiratory mucosa from a 19-day rat fetus. The explant was cultured 4 days. Orig. mag. 360×. Fig. 11: Explant of masticatory mucosa from the hard palate of a 15-day rat fetus after 1 day of culture. The arrow points to an area of sheetlike epithelial outgrowth. Orig. mag. 35×. Fig. 12: Same explant as in Fig. 11 after 4 days of culture. The arrow points to the same area. Note the epithelial repair. Orig. mag. 35×.

all of which had variations in the medium and culturing techniques. From the various media used in culturing skin and oral mucous membrane, it is obvious that the organ explants retain their selective nutritional capabilities.[8]

The medium contained thrombin to facilitate clotting, although the clot did not always form. It was found that after 30 minutes of incubation, if an additional 0.1–0.2 cc. of the balanced salt solution containing thrombin was added to the medium, coagulation occurred.

Storing the fetuses in balanced salt solution before dissection had no effect on the growth of the explants. Some tissues were dissected within a few minutes after securing the fetal heads. The last dissections of the same litter were made about 6 hours later. The viability of the tissue was not altered during this waiting period, nor were there any obvious differences between results of either the first or the last dissection.

The maximum outgrowth occurred in 96 hours (Fig. 2). Where outgrowth occurred, it was removed, and the explant was subcultured. However, some explants had little, if any, outgrowth.

Whether the outgrowth was removed or not, histologic examination showed repair of the submucosa by epithelial covering. This is a primary function of the oral mucosa. When no outgrowth occurred, histologic examination established that the epithelium covered the submucosa and matured.

Sheetlike outgrowth in the cultivation of skin has already been reported.[3-7] This was the predominant type of growth in masticatory epithelium.

Some budding outgrowth was also noted in masticatory mucosa. The budding was much less than that observed in tooth buds. Tonguelike (budding) growth patterns from skin occurred with the regeneration of sebaceous glands and hair follicles.[12] The similar type of outgrowth, noted in masticatory mucosa, is possibly related to palatal gland formation.

Dental epithelium is that portion of the oral epithelium that is associated with the formation of the tooth. It is also the residual epithelium found after tooth formation.[2] This type of epithelium plays an important part in the formation of odontogenic cysts, tumors of the oral region, and periodontal disease.

Dental epithelium has a budding growth pattern. Both intact tooth buds and tooth buds with the outer tunic removed exhibited the budding proliferation (Fig. 8).

Respiratory mucosa grew with a sheetlike epithelial proliferation (Fig. 9). This has previously been reported.[10] This sheetlike growth pattern is as would be expected. One function of respiratory epithelium is to cover or line the respiratory cavities. The respiratory epithelium also became ciliated and formed goblet cells. This type of epithelium may enter into the formation of certain non-odontogenic cysts of the oral region.

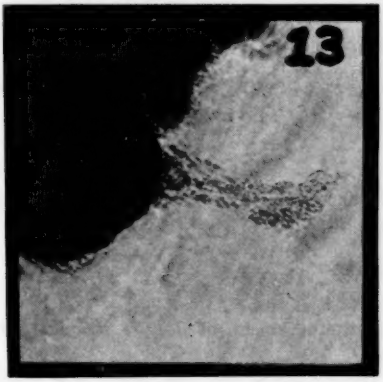
An interesting additional type of growth pattern was observed in explants of both masticatory mucosa and respiratory mucosa. It took the form of a large, bulbous outgrowth which stemmed from areas of sheetlike growth (Figs. 11 and 12).

On the basis of gross examination, shrinkage of the explant occurred shortly after planting. In addition, there was also retraction of the outgrowth (Figs. 13 and 14). Retraction of outgrowth has been observed in cultures of skin.[2, 3]

The problem of maturation in vitro was a primary objective of this study. With this in mind, masticatory mucosa from 15-day fetuses was cultured because it exhibited no differentiation and no keratinization.[11] From an epithelium consisting of three to four

cell layers (Fig. 1) differentiation and maturation progressed to the point of keratinization (Fig. 4). Growth and differentiation to the point of keratinization had previously been accomplished in cultures of skin.[13-20]

Maturation is slightly more rapid in vitro than in vivo. Keratinization in one explant occurred after 2 days (Fig. 3). An increased rate of keratinization in vitro in



FIGS. 13–14.—Fig. 13: Budding outgrowth in an explant of a tooth bud from a 19-day rat fetus after 1 day of culture. Orig. mag. 100×. Fig. 14: Same area as in Fig. 13 after 2 days of culture. Shrinkage of the whole explant has occurred, as well as retraction of the budding epithelial outgrowth. Orig. mag. 100×.

TABLE 2

Tissue	Days of Growth	Outgrowth		Histologic Observations
		Epithelium	Fibroblasts	
Palatal mucosa, 15-day rat fetus	1	Slight, sheetlike, irregular	.....	After 2 days: thin layer of keratin
	4	Increased	Slight	After 4 days: tissue rounded up into a ball of connective tissue covered with mature keratinized epithelium
	8	Very little outgrowth		
Palatal mucosa, 19-day rat fetus	1	Slight	.....	Tissue matured fully in 2 days
	10	Minimum, sheetlike	Minimum	
Ridge mucosa, 19-day rat fetus	1	Present	None	Same as above
	2–6	Progressive increase, sheetlike and some budding	Present	
Dental Epithelium, 19-day rat fetus				
Entire tooth bud	4	Budding	.....	Mass of epithelium with central area of organization, pre-ameloblasts
Without outer tunic	4	Budding	Slight	
Respiratory mucosa, 15-day rat fetus	1	Sheetlike	Present	Mature, pseudo-stratified, ciliated, columnar epithelium; no goblet cells
Respiratory mucosa, 19-day rat fetus	4	Sheetlike	Slight	Mature respiratory epithelium, pseudo-stratified and ciliated; goblet cells

cultures of skin has been attributed to a vitamin A deficiency.[21] Excessive vitamin A suppressed keratinization.[22] (In this investigation, no analysis of vitamin A was made.)

In one explant an epithelial pearl formed; this is described as "dyskeratosis." The basal cells were on the outer surface of the explant; the central cavity was filled with keratin. Similar development has been observed in cultures of skin and tooth buds.[22-24]

SUMMARY

Table 2 summarizes the results of this study. Masticatory mucosa, dental epithelium, and respiratory mucosa develop and mature in vitro. The results offer an experimental method for further study of these tissues.

The author wishes to express his grateful acknowledgment to Dr. Hamilton, B. G. Robinson, and Dr. William Lefkowitz for their invaluable assistance in this study.

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The Action of Enymes on Ethylene Oxide–treated Periodontal Tissue of Rats

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Evidence has been accumulating to support the concept that bacterial enzymes may be intimately related to periodontal disease. It has been shown that organisms from human gingiva are able to produce, in vitro, enzymes that are destructive to tissues or to certain chemical and structural components of tissues.[1-8] Particular attention has been directed toward the effects of enzymes on the ground substance and on collagen. A number of investigators have studied enzymatic digestion of collagen fibers[9-13] and have reported that the rate of lysis may vary, depending upon the amount of tension applied to the fibers.

The present report concerns a study of the effects of bacterial enzymes and selected pure enzymes on the periodontal membrane of rat incisors which, after removal of the jaws and preparation in the laboratory, were subjected to tension. Theoretically, tension in the fibers could cause separation of component fibrils, or tactoids, making them more accessible to enzyme action. The resultant destruction to the fibers would result in extraction of the stressed tooth or could be evaluated histologically.

MATERIALS AND METHODS

Adult white rats were sacrificed by an interperitoneal injection of sodium pentothal. A block of bone and tissue containing either maxillary or manidbular incisor teeth was dissected out and prepared so that one pin clamp could be placed in the pulp canal and another through the supporting bone, leaving the tooth suspended only by the periodontal fibers. The specimens as prepared were approximately 10 mm. in length and represented the unerupted segment of the tooth with the incisal and apical portions removed. This served as an ***in situ*** in vitro experimental unit (Fig. 1).

Prior to incubating the experimental unit with the various bacterial cultures or pure enzymes, it was necessary to sterilize the unit. This was accomplished by two methods. In one, the tissue was placed in 100 per cent ethylene oxide(liquid) at 0° C. for 2 hours. It was then air-dried at room temperature for 20 minutes. All concentrations of ethylene oxide below this amount resulted in sterilization of surface organisms only. In the second method the tissue was placed in 4 per cent formaldehyde for 4 hours, then washed in sterile water for 1 hour. Following sterilization, the tissue was attached to the weighted unit. In Phase I a weight was applied which exerted just enough tension to

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keep the specimen immersed in the medium. In Phase II the weight was 4.5 gm., and in Phase III it was 9.5 gm. These forces were not intended to simulate the natural forces exerted on the periodontal fibers during function but rather were intended to see whether stretching would open the complicated protein molecule chain of collagen and thus make areas available for penetration by the enzymes.

Both thioglycollate and nutrient broth* were used. The unit was inoculated with

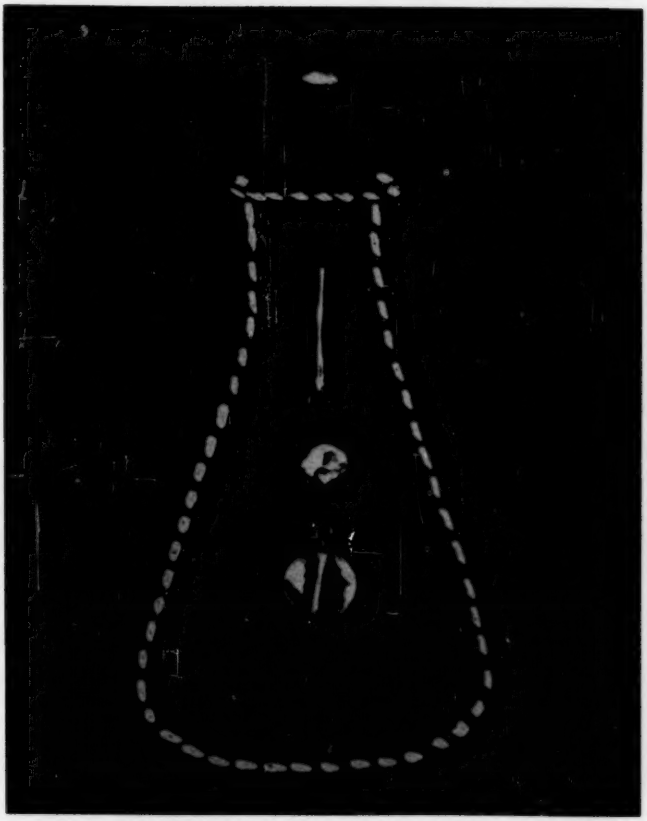

FIG. 1.—Arrangement of *in situ, in vitro* experimental unit showing disk-shaped weight suspended from block of tissue.

known suspensions of pure cultures or with cultures taken on sterile paper points from the inflamed gingival sulci of human subjects (Table 1). Incubation was at 37° C. for 48 hours. The controls were similarly prepared and incubated in sterile media. One maxillary or mandibular incisor was used as the experimental unit, with the tooth from the opposite side serving as its control. The pure cultures used were Nocardia D10B and XD6A secured from carious teeth[14] and known to produce proteolytic enzymes. Cultures of Tetrads 136E and 160A, also proteolytic and isolated from periodontal pockets,[8] were employed.

* Manufactured by The Difco Manufacturing Co.

Proteolytic enzymes were selected for the enzyme series. An antibiotic (penicillin and dihydrostreptomycin 400/0.5*) was added to each unit to insure sterility of the enzyme itself. The antibiotic was also added to each control. The medium was thioglycollate broth, and in this series the heaviest weight (Phase III) was attached. Incubation was at 37° C. for 72 hours (Table 2).

After incubation all tissue was fixed in 4 per cent formaldehyde for 27 hours, followed by decalcification by ethylenediaminetetraacetic acid tetrasodium salt at pH 7.4 for 7 days. Decalcification as verified by X-rays was usually complete at this time. If an extraction had occurred, the tooth was replaced in its socket during paraffin imbedding. Cross-sections of the teeth were taken from varying depths of the specimen. These sections were stained with Harris' hemotoxylin and eosin, Van Gieson's, and Mallory.

TABLE 1

BACTERIAL STUDIES

Ethylene Oxide Sterilization*	Mixed Culture	Nocardia	Tetrads	Total Experimental Units	Total Control Units
1. Thioglycollate broth					
Phase I, tension only	4	4	4	12	12
Phase II, 4.5-gm. weight	2	4	4	10	10
Phase III, 9.5-gm. weight	2	4	4	10	10
2. Nutrient broth					
Phase I, tension only	4	4	4	12	12
Phase II, 4.5-gm. weight	2	4	4	10	10
Phase III, 9.5-gm. weight	2	4	4	10	10
Total	16	24	24	64	64

* Formaldehyde sterilization was a duplication of the ethylene oxide sterilization.

TABLE 2

ENZYME STUDIES*

Ethylene Oxide Sterilization†	Experimental Units	Control Units
Enzymes:		
25 mg. trypsin 2X cryst. (50% $MgSO_4$)	2	2
25 mg. uricase (semipurified)	2	2
25 mg. cathepsin (beef spleen) without A and B	2	2
25 mg. papain powder	2	2
25 mg. trypsin 4X U.S.P.	2	2
25 mg. collagenase	2	2
Total	12	12

* Medium: thioglycollate broth (durycin F.A. [Lily] U.S.P.; penicillin and dihydrostreptomycin 400/0.5 added to each unit), 52 cc.; incubation: 3 days at 37° C.; weights: 9.5 gm.

† Formaldehyde Sterilization was a duplication of the ethylene oxide sterilization.

* Durycin, manufactured by Eli Lilly and Co.

RESULTS

A. *Formalin-sterilized tissue.*—Tissues which were presterilized with formaldehyde, then incubated with either pure cultures of Nocardia, Tetrads, or mixed cultures, showed no differences between the experimental and control series, regardless of the amount of weight applied. Proteolytic enzymes tested were also inactive. Figure 2 shows an area of the periodontal membrane of a control mandible from this series. Here the fibers show an orderly arrangement and are well attached to both alveolar bone and cementum. The sinuous course of the fibers is a feature of the periodontal membrane of the continuously erupting incisor tooth.

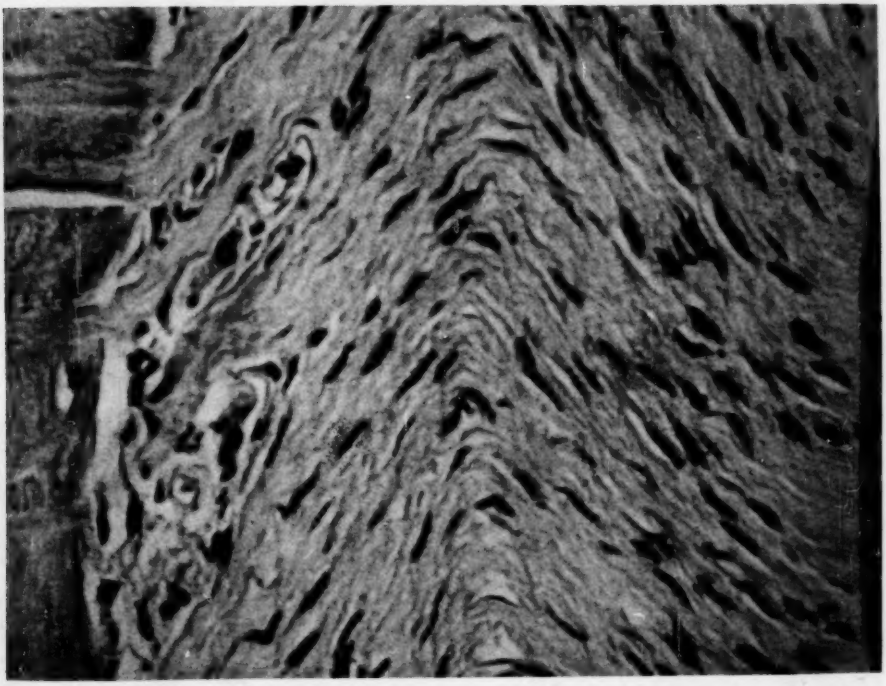

FIG. 2.—(6 cm) Section from mandible of control specimen sterilized in formaldehyde (×300)

B. *Ethylene oxide–sterilized tissue.*—In a preliminary study to determine the amount of ethylene oxide necessary to sterilize the tissue block, all amounts under that finally established (100 per cent) resulted in bacterial growth in the unit and rapid extraction of the teeth.

Following sterilization with ethylene oxide, the controls (example, Fig. 3) showed, in general, a separation of the periodontal fibers by vacuoles which made them more distinct in their outlines. Toward the alveolar bone there were seen, in addition to vacuoles, vascular spaces representing blood vesesls of the periodontal membrane. The larger bundles of collagenous fibers were intact in their attachment to the cementum. Vacuolization was not invariably as severe as this.

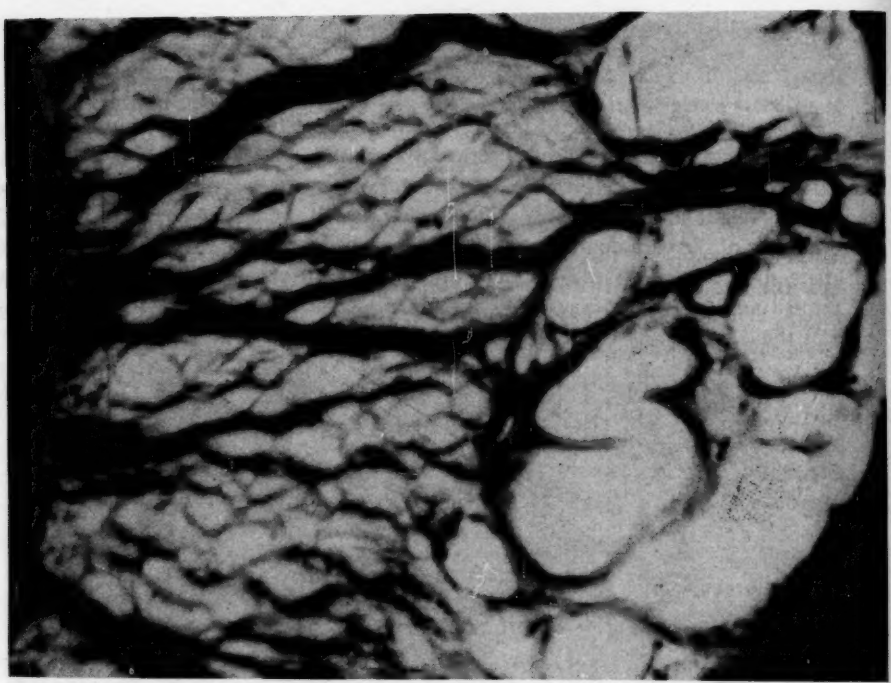

FIG. 3.—(Δ7 clm) Section from mandible of control specimen sterilized in ethylene oxide (×300). Vacuolization between fibers occurred characteristically as shown here but was not invariably present to this degree (see Fig. 4).

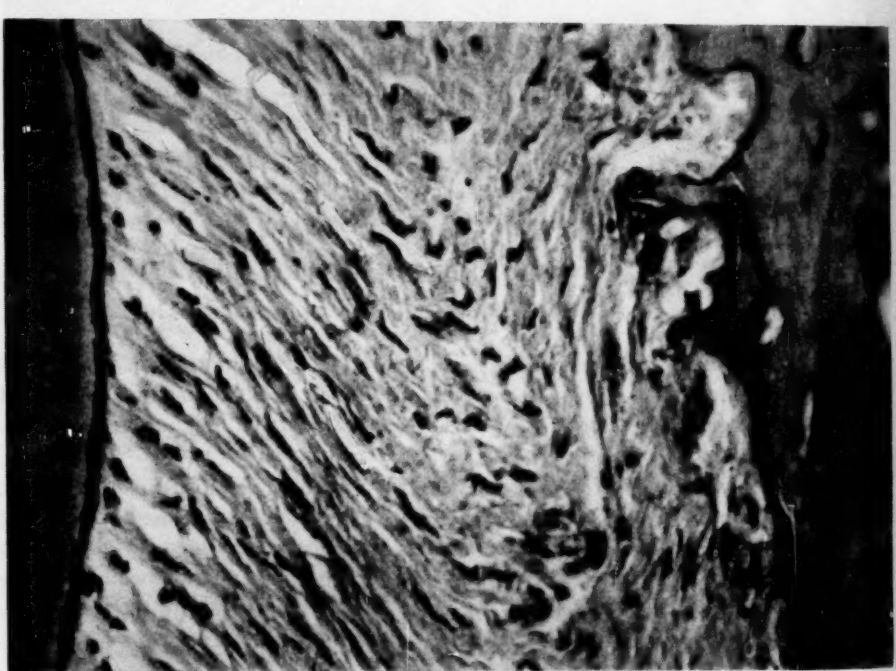

FIG. 4.—(Δ2 cm) Section from mandible of control specimen sterilized in ethylene oxide and incubated in medium with antibiotic only. In this specimen vacuolization is very slight (×300).

In the bacterial experimental series the addition of pure cultures or mixed cultures caused no discernible differences between experimental and control sections.

In the enzyme series the addition of the antibiotic caused no apparent changes in the controls (Fig. 4). All enzymes added caused either extractions of the teeth or destruction that was visible in the histological study.

Uricase treatment resulted in no extractions. Cellular detail was still present, and the fibers retained a fairly orderly arrangement. Fragments of chromatin suggesting disintegration were seen. There was disorientation of fibers near the bone, where there was, in addition, moderate hyalinization and loss of attachment.

Trypsin 2X caused extraction of the teeth. There was destruction of the cells of the bone and cementum. One control tooth in this series was extracted, and the histological picture showed rupture of the mid-section of the fibers with little alteration of cellular detail.

Trypsin 4X also resulted in extraction of the teeth. Here it was observed that cellular destruction was marked.

Papain caused extraction of the teeth. The remaining fragments of the periodontal membrane appeared threadlike (Fig. 5).

Cathepsin, although causing extraction of the mandibular tooth and fragmentation of the periodontal membrane, did not cause extraction of the maxillary tooth tested. Some nuclei were still present in both sections, indicating lesser destructive activity than that produced by other enzymes.

Collagenase caused complete destruction of the soft-tissue elements, with extraction of the teeth. Here more destruction was seen than in any other unit.

DISCUSSION

The tissue presterilized with formaldehyde showed no alteration, regardless of the type of experimental procedure. Although the formaldehyde was effective in sterilization, it presented the problem of fixation of tissues. Such fixed tissues might be assumed to be less permeable to enzymatic action, although Stoughton[15] reported enzymatic cytolysis of tissues which had been formalin-fixed.

Tissue presterilized with ethylene oxide suffered some change as a result of this treatment as indicated by the histological study. Vacuolization occurred which tended to separate the periodontal membrane fibers. The fibers often appeared slender and somewhat "angular." Thonard and Scherp[6] have described changes in collagen in the gingival tissues and bovine Achilles' tendon treated with ethylene oxide and stained with Mallory stain. We did not see the swelling and homogenizaiton of fibers which they describe. However, their method differs from the procedure in this experiment, in that they used frozen collagen, then ethylene oxide vapor for 18 hours at room temperature.

Lysis of tissue elements as portrayed by extraction of the teeth or by histological studies indicated that when the concentration of certain pure enzymes was sufficient, changes would result. The pure cultures and mixed cultures tested were not able to evoke any changes in the experiment.

SUMMARY

An *in situ* in vitro unit has been devised to study the effects of certain enzymes on collagen of periodontal membrane fibers of rat incisors that had been sterilized in

formalin or ethylene oxide. Other investigators have studied enzymatic digestion of collagen following the use of these reagents and have found alterations in the rate of lysis, depending on the amount of tension applied to the fibers.

By means of weights applied to the incisors *in situ*, the teeth were placed under tension of varying amounts. The units were sterilized with either formalin or ethylene oxide and incubated in cultures of proteolytic organisms or in solutions of pure enzymes (see Tables 1 and 2). A total of 152 experimental and 152 control units has been studied.

FIG. 5.—(Δ4 TM) Severe destruction of the periodontal membrane following incubation with papain. A similar degree of tissue damage was wrought by collagenase (×300).

No destruction of collagen was evident in experimental or control tissue sterilized in formalin, contrary to other reports.[15] Sterilization with ethylene oxide altered the appearance of the periodontal membrane fibers, but the fibers were not observably altered thereafter by incubation in bacterial cultures. The purified enzymes tested (trypsin 2X, 4X, papain, cathepsin, uricase, and collagenase) caused varying degrees of destruction of the fibrous elements. Uricase was least active.

No alterations were observed that could be attributed directly to tension produced by increasing the weight load. The findings indicate that purified enzymes in sufficient concentration may affect periodontal membrane fibers and that the effect may be modified by fixation of the tissue.

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The Solution of Calcium and Phosphorus from the Intact Enamel Surface

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Although the solubility of the dental enamel has been the subject of many investigations, both qualitative and quantitative, surprisingly little work has been done on the intact enamel surface. Because the surface enamel is the layer primarily concerned during an acid attack, it seems reasonable to suppose that a study of the solubility of this region would yield information on the way in which the attack is initiated. The majority of findings, however, relate to ground enamel or specially prepared surfaces, and even where the intact enamel surface has been tested, most investigators—apart from Hardwick[1] and Brudevold and Little[2]—have reported solubilities in terms of either calcium dissolved or phosphorus dissolved, but not both.

In the experiments described below, an attempt has been made to measure the rate of loss of both calcium and phosphorus from the intact enamel surface over a range of pH values. These experiments are part of a series being carried out on the enamel surface in the hope that a clearer picture of the mechanism of enamel solution will emerge from the results.

EXPERIMENTAL METHODS

In the method used to measure the rate of enamel solution, teeth were masked with wax, except for a small regular area of enamel surface of known dimensions. Each tooth was placed in a buffered solution of acid and continuously shaken at a carefully controlled temperature for a standard length of time. A portion of the acid solution was then analyzed for calcium and phosphorus.

The technique used to mask the freshly extracted incisors employed in the experiments was that devised by Brudevold.[3] A circular area of labial enamel, 2 mm. in diameter, was exposed on each tooth. Into a boiling tube were placed a measured volume of N hydrochloric acid–sodium acetate buffer and the mounted tooth, and the tube and its contents were shaken at 37° C. for the required length of time. At the end of this period the tooth was removed from the tube, and the amounts of calcium and phosphorus which had dissolved from the enamel surface were measured by chemical analysis of the solution.

As the amount of enamel dissolved was often only a few micrograms, it was found necessary to use micromethods for the determination of both calcium and phosphorus. Calcium was precipitated as the oxalate, which was then titrated with 0.01 N potassium permanganate. The microtitrations were carried out with the aid of the micrometer

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syringe, and, from estimations made on standard solutions, it was found that calcium concentrations of 10 μg/ml and even less could be determined with an error of not more than ± 2 per cent. Phosphorus was estimated by the method of Kuttner and Cohen.[4] The error of phosphorus determinations was found to be ± 1 per cent.

Experiments were carried out on three groups of teeth, each group comprising fifty incisors. Each tooth in the first group was shaken for 1 hour with 10 ml. of acid buffer at pH 2.0. In the second group each tooth was shaken with 5 ml. of buffer at pH 4.0 for 1 hour. In the last group each incisor was shaken for 2 hours with 5 ml. of buffer at pH 5.0. The longer period of shaking used in the experiments at pH 5.0 is needed to dissolve measurable amounts of calcium from the enamel surface.

TABLE 1

CALCIUM AND PHOSPHORUS SOLUBILITY OF SURFACE ENAMEL AT pH 2.0

Tooth No.	Ca (μg)	P (μg)	Ca/P	Tooth No.	Ca (μg)	P (μg)	Ca/P
1	407	169	2.41	26	534	236	2.27
2	461	182	2.54	27	352	160	2.20
3	712	283	2.52	28	347	159	2.18
4	825	360	2.29	29	338	158	2.14
5	759	347	2.19	30	311	146	2.13
6	458	246	1.86	31	624	270	2.32
7	180	95	1.89	32	619	265	2.34
8	425	192	2.22	33	931	403	2.31
9	446	210	2.12	34	515	222	2.32
10	399	182	2.19	35	823	340	2.42
11	339	148	2.30	36	521	225	2.31
12	482	200	2.41	37	357	154	2.32
13	1,141	466	2.45	38	404	176	2.30
14	624	272	2.30	39	515	220	2.34
15	626	276	2.27	40	379	158	2.40
16	635	284	2.24	41	586	256	2.29
17	478	206	2.32	42	555	248	2.24
18	389	169	2.30	43	644	275	2.34
19	430	183	2.35	44	498	212	2.35
20	347	140	2.49	45	659	276	2.38
21	652	287	2.27	46	458	202	2.27
22	574	235	2.44	47	417	173	2.41
23	680	300	2.27	48	596	260	2.29
24	448	196	2.29	49	483	216	2.24
25	744	340	2.19	50	508	214	2.37

RESULTS

For each individual enamel area, the total losses of calcium and phosphorus are recorded in Tables 1, 2, and 3. The ratio of dissolved calcium to dissolved phosphorus is also calculated in each case.

The solubilities show a good deal of variation, although the solubility ranges at pH values 2.0 and 4.0 are much smaller than that at pH 5.0. The highest value of the calcium solubility at pH 2.0 is about six times as great as the lowest value, and at pH 4.0 the ratio is about three to one. At pH 5.0, on the other hand, the highest calcium solubility recorded is more than forty times as great as the lowest.

The range of values of phosphorus solubility at each pH value is also fairly large. At

TABLE 2

Calcium and Phosphorus Solubility of Surface Enamel at pH 4.0

Tooth No.	Ca (μg)	P (μg)	Ca/P	Tooth No.	Ca (μg)	P (μg)	Ca/P
52	88.1	42.5	2.07	77	75.2	34.6	2.17
53	179.8	77.5	2.32	78	95.4	41.0	2.33
54	74.6	36.6	2.04	79	77.0	30.9	2.49
55	95.9	45.7	2.10	80	67.7	30.5	2.22
56	121.6	56.4	2.16	81	99.4	41.0	2.42
57	94.7	47.0	2.01	82	91.7	45.9	2.00
58	80.1	36.8	2.18	83	74.0	30.8	2.40
59	109.8	56.0	1.96	84	58.8	24.9	2.36
61	86.7	36.6	2.37	85	112.1	49.7	2.26
62	114.0	50.2	2.27	86	74.8	27.5	2.72
63	70.2	29.0	2.42	87	69.7	30.2	2.30
64	86.8	31.5	2.76	88	88.5	37.4	2.37
65	78.9	33.8	2.33	89	77.6	30.8	2.52
66	73.0	30.7	2.38	90	92.3	40.2	2.30
67	53.9	23.0	2.34	91	76.4	26.7	2.86
68	73.3	30.5	2.40	92	86.9	23.0	3.78
69	62.5	23.7	2.64	93	106.5	40.5	2.63
70	62.6	28.0	2.24	94	119.6	46.4	2.58
71	79.0	35.1	2.25	95	94.2	38.7	2.43
72	62.0	28.6	2.17	96	80.6	33.2	2.43
73	76.7	30.4	2.52	97	104.3	36.8	2.83
74	87.5	40.8	2.14	98	69.1	25.3	2.73
75	66.3	27.9	2.38	99	86.5	34.1	2.54
76	91.7	37.4	2.45	100	96.8	30.8	3.14

TABLE 3

Calcium and Phosphorus Solubility of Surface Enamel at pH 5.0

Tooth No.	Ca (μg)	P (μg)	Ca/P	Tooth No.	Ca (μg)	P (μg)	Ca/P
101	69.1	26.8	2.58	126	19.8	15.8	1.25
102	80.0	16.4	4.88	127	33.4	20.3	1.65
103	94.6	43.0	2.20	128	31.3	17.1	1.83
104	108.9	41.0	2.66	129	70.5	21.8	3.23
105	36.4	26.5	1.37	130	15.8	10.5	1.50
106	65.7	21.4	3.07	131	31.1	13.1	2.37
107	9.3	11.6	0.80	132	44.7	21.0	2.13
108	35.8	22.2	1.61	133	48.1	21.2	2.27
109	110.7	55.4	2.00	134	50.5	14.0	3.61
110	17.0	12.8	1.33	135	42.9	19.7	2.18
111	39.6	26.8	1.48	136	40.2	16.9	2.38
112	64.9	30.3	2.14	137	28.5	11.8	2.42
113	28.3	18.5	1.53	138	20.8	10.8	1.93
114	51.5	19.0	2.71	139	107.9	16.4	7.00
115	47.1	29.2	1.61	140	41.0	17.5	2.34
116	27.7	16.0	1.73	141	8.7	17.5	0.50
117	54.6	28.3	1.93	142	12.1	15.0	0.81
118	42.6	16.5	2.58	143	5.5	14.4	0.38
119	184.6	36.8	5.02	144	49.9	31.5	1.58
120	23.7	18.0	1.32	145	11.9	16.4	0.73
121	60.8	24.3	2.50	146	8.7	17.4	0.50
122	48.3	24.2	2.00	147	5.1	10.0	0.51
123	70.1	26.9	2.61	148	4.5	10.4	0.43
124	37.6	20.0	1.88	149	80.4	11.0	7.31
125	27.5	17.1	1.61	150	4.5	11.7	0.39

pH levels 2.0 and 4.0, the highest phosphorus solubilities are five and three times as great, respectively, as the lowest solubilities. At pH 5.0 the range of phosphorus solubilities is much smaller than the corresponding range of calcium solubilities, although the ratio of highest to lowest values is still about five to one.

Thus, on the whole, it may be stated that calcium and phosphorus show a similar range of solubilities at both pH 2.0 and pH 4.0, but at pH 5.0 the range of values of calcium solubility is much greater than the range of values of phosphorus solubility. This state of affairs is shown more clearly, perhaps, by the variation in the Ca/P ratio calculated for each individual experiment. At pH levels 2.0 and 4.0, the range in values

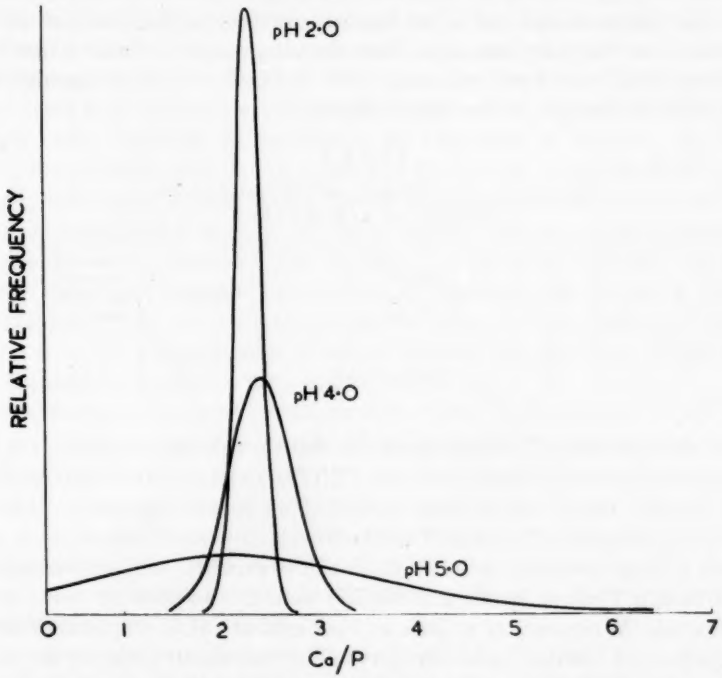

FIG. 1.—Distribution of the dissolved calcium to dissolved phosphorus ratios at pH 2.0, 4.0, and 5.0

of the Ca/P ratio is fairly small, but at pH 5.0 the values of the Ca/P ratio are scattered over a range from 0.38 to 7.00 (see Fig. 1).

The mean value of the Ca/P ratio at different pH levels, however, do not differ greatly. The mean Ca/P ratios (calculated from the total calcium and phosphorus solubilities of each group of fifty teeth) at pH values 2.0, 4.0, and 5.0 are 2.30, 2.37, and 2.19, respectively.

DISCUSSION

Inspection of Figure 1 and the contents of Tables 1, 2, and 3 indicates that there is one salient feature of the results which warrants closer examination. Whereas the ratios of dissolved calcium to dissolved phosphorus for individual teeth are scattered over a large range of values at pH 5.0, the ratios at pH levels 2.0 and 4.0 fall within a fairly

narrow range of values. The reason for this difference may be found in the chemical structure of the tooth, but before further discussion of this point is possible, it is first necessary to touch briefly on the chemical composition of the dental enamel.

Although the amounts of the principal constituents of the enamel found by different investigators are subject to a certain amount of variation, on the whole the results show good agreement. The figures in Table 4 are the percentage values found by Armstrong and Brekhus[5] for the principal constituents of enamel from caries-free permanent human teeth after fat extraction with alcohol-ether and careful drying.

The analyses were carried out on the combined enamel of many teeth, and the figures obtained may therefore be regarded as average values for dental enamel. If it is assumed that all the phosphorus of the enamel is present as PO_4''' and all the CO_2 as CO_3'', then it may be easily calculated from the values given in Table 4 that $PO_4''' =$ 53.7 per cent, $CO_3'' = 4.1$ per cent, and $Ca^{++} + PO_4''' + CO_3'' =$ approximately 93 per cent of the total weight of the dental enamel.

TABLE 4

PRINCIPAL CONSTITUENTS OF DENTAL ENAMEL
(ARMSTRONG AND BREKHUS)

	Per Cent	Standard Deviation (Per Cent)		Per Cent	Standard Deviation (Per Cent)
Ca.....	35.41	0.96	CO_2.....	3.00	0.24
P.....	17.45	0.51	Mg.....	0.30	0.05

Most of the experimental evidence indicates that the calcium phosphate, $Ca_3(PO_4)_2$, of the enamel is present as hydroxyapatite, $Ca_5(PO_4)_3OH$; a number of investigators, including Thewlis, Glock, and Murray[6] and Tovborg Jensen and Møller,[7] have found that the lattice dimensions for enamel and hydroxyapatite are identical. Thus the dental enamel is most probably a mixture of hydroxyapatite and calcium carbonate, $CaCO_3$. Although Tovborg Jensen and Møller[7] were able to show by X-ray crystallographic methods the presence of as little as 1 per cent of $CaCO_3$ in powder mixtures of hydroxyapatite and calcium carbonate (crystallized as calcite), they were unable to obtain any lines indicating the presence of calcite in dental enamel. It was assumed, therefore, that the calcium carbonate of the enamel exists in the amorphous form, not as part of the apatite lattice, but occluded interstitially, or bound adsorptively to the surface of the apatite crystallites.

The Ca/P ratio of hydroxyapatite is 2.15, and the average Ca/P ratio for 42 samples of enamel examined by Armstrong and Brekhus[5] is 2.03. Thus there is present in the enamel a certain amount of phosphate in excess of that required by the hydroxyapatite formula. The ability of hydroxyapatite to adsorb phosphate ions has been recognized for some time, but Hodge *et al.*[8] showed that enamel adsorbs phosphates from aqueous solutions at 40° C. in a manner comparable to the adsorption of phosphates by hydroxyapatite. Posner and Stephenson[9] found that hydroxyapatite which had adsorbed up to 6 per cent by weight of phosphate ion gave no indication when examined by X-ray crystallographic methods that adsorbed phosphate existed in a developed crystalline state. From this and other data it was concluded that, in dental enamel, excess

phosphate, like calcium carbonate, is adsorptively bound to the surface of the apatite crystallites. On the other hand, more recent work carried out by Posner and Perloff[10] on the structure of synthetic "defect" apatites indicates that the low Ca/P ratio of dental enamel may be due to a statistical absence of calcium ions from structural positions throughout the hydroxyapatite crystal rather than to an excess of adsorbed phosphate.

On the basis of the foregoing findings, it is now possible to offer a tentative explanation of the large range of values of the dissolved calcium to dissolved phosphorus ratio at pH 5.0. It is necessary, however, to make two assumptions. First, it must be supposed that the rate of release of Ca in the initial stages of an acid attack is much higher from an enamel containing a high percentage of $CaCO_3$ than from an enamel which contains little carbonate. Some support for this supposition may be derived from the work of Logan and Taylor,[11] who found that, by means of continuous addition of dilute acid to the solid, more than 50 per cent of the $CaCO_3$ can be removed from the glycerol ash of bone, while less than 10 per cent of the phosphate is removed. Second, it is necessary to assume that the $CaCO_3$ content of the enamel is not constant but varies considerably from tooth to tooth. This is hardly a rash assumption to make, as is shown by the range of estimates of the CO_2 content of enamel made by different investigators. For instance, Bowes and Murray[12] and Bremer[13] give values of 1.95 and 3.66 per cent, respectively, for the CO_2 content, although their values for the Ca and P content of enamel agree closely. It is important to note that each of these figures represents the mean CO_2 content of a large number of enamel samples and, therefore, the variation in CO_2 content between individual teeth will be much larger.

Now consider the solution of individual areas of the surface enamel at pH 5.0. Because of the relatively high pH, the rate of solution will be slow, and the amount of enamel dissolved will be small. Moreover, the duration of the experiment is only a fraction of the time needed for the exchange of ions betwen solid and liquid phases to reach equilibrium. Under such conditions, the amount of occluded calcium carbonate and the presence of the excess adsorbed phosphate may exert great influence on the relative amounts of Ca and P which go into solution. We may conveniently divide the individual enamels into three classes.

1. *The enamels contain a high percentage of $CaCO_3$.*—Because $CaCO_3$ is more soluble in hydrochloric acid solution than hydroxyapatite is, it is likely to be dissolved preferentially. The removal, by solution, of the occluded $CaCO_3$ will tend to increase the surface area of hydroxyapatite crystals in contact with the solution and promote more rapid solution of the apatite $Ca_3(PO_4)_2$. The amount of hydroxyapatite dissolved during the experimental period, however, should always be much smaller than the amount of $CaCO_3$ dissolved, and the net result will be that the total weight of Ca dissolved from $CaCO_3$ and hydroxyapatite should be much greater than the total weight of P dissolved from the apatite and the adsorbed phosphate. Thus, when the loss of calcium is high, the ratio of dissolved Ca to dissolved P should also be high.

2. *The enamels contain a low percentage of $CaCO_3$.*—As the $CaCO_3$ present releases only a small quantity and the apatite a negligible amount of Ca to the solution during the experimental period, then the excess adsorbed phosphate, which goes easily into the solution, should have a large effect on the ratio of dissolved Ca to dissolved P. In other words, the total weight of P released from adsorbed phosphate and hydroxyapatite

should be as great as, or greater than, the total weight of Ca released from $CaCO_3$ and hydroxyapatite. Therefore, when the loss of calcium is low, the ratio of dissolved Ca to dissolved P should also be low. If the defect structure of Posner and Perloff[10] is assumed for enamel, then an enamel low in carbonate would also tend to give a low dissolved Ca/P ratio, although without the release of adsorbed phosphate it is difficult to postulate a hydroxyapatite structure with a deficiency of Ca ions great enough to produce some of the very low dissolved Ca/P ratios (e.g., 0.38) found experimentally.

3. *The enamels contain percentages of $CaCO_3$ intermediate between those of enamels 1 and 2.*—The bulk of individual enamels will fall into this class and should show a

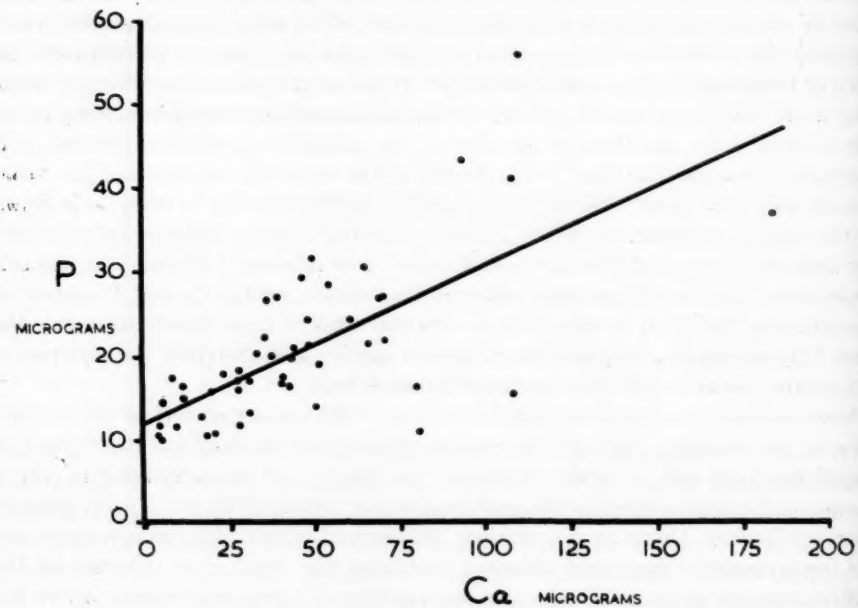

FIG. 2.—Scatter diagram relating Ca and P solubility at pH 5.0

gradation of values for the loss of calcium and the dissolved Ca/P ratio intermediate between those given by enamels 1 and 2. The Ca/P ratio should still depend upon the total Ca dissolved.

If the experimental values at pH 5.0 of dissolved Ca are plotted against the values of dissolved P, the graph shown in Figure 2 is obtained.

The straight line which is the best fit to the points on the graph was calculated from the Ca and P values by the method of least squares. The correlation between the variables P and Ca is positive and highly significant ($r = 0.68$; Probability < 0.001). Now the equation for the line of best fit is

$$P = a + b\text{Ca} . \tag{1}$$

where a = intercept on the P axis = 12.5 and

$$b = \text{slope} = 0.18 .$$

Equation (1) may be written:

$$P = 12.5 + 0.18Ca\ . \tag{2}$$

If both sides of equation (2) are divided by Ca, then

$$\frac{P}{Ca} = \frac{12.5}{Ca} + 0.18\ . \tag{3}$$

From equation (3) it can be seen that

(*a*) When Ca is large, P/Ca is small, or Ca/P is great.

(*b*) When Ca is small, P/Ca is great, or Ca/P is small.

Equation (2) indicates that when Ca = 0, i.e., even when no calcium is dissolved, a certain amount of phosphorus goes into solution. It may be shown that for the enamel area used in each experiment (*ca.* 0.03 cm.^{2}) this amount of phosphorus is equal to 12.5 ± 3.2 μg.

The theory previously put forward to account for the large range in values at pH 5.0 of the Ca/P ratio would appear to agree with the experimental findings. Thus the theory predicts and the facts tend to show the following conclusions:

1. If solution of calcium from tooth A is greater than solution of calcium from tooth B, then the loss of phosphorus from A is also greater than the loss of phosphorus from B.

2. The ratio of dissolved Ca to dissolved P depends upon the amount of Ca dissolved from the enamel. If the Ca solubility is high, the Ca/P ratio also tends to be high, and if the Ca solubility is low, then the Ca/P ratio is also low.

3. There is an excess of phosphate in the enamel which is not bound to calcium and which goes into solution quite independently of the Ca and P of hydroxyapatite. Even if no calcium is dissolved from the enamel, some phosphate will find its way into solution.

Similar, though smaller, effects are produced during the solution of enamel at pH 4.0 and pH 2.0. The dissolved Ca/P ratios range from 1.96 to 3.78 at pH 4.0 and from 1.86 to 2.54 at pH 2.0. The greater solution of enamel at these lower pH values, especially at pH 2.0, tends to swamp the effect that the $CaCO_3$ and adsorbed phosphate have upon the Ca/P ratio. Consequently, the range of values of the dissolved Ca/P ratio becomes smaller as the pH falls.

SUMMARY

Incisor teeth covered with wax, except for a small area of the labial enamel surface, were shaken individually with buffered acid solutions at 37° C. over a range of pH from 2.0 to 5.0. The amounts of calcium and phosphorus dissolved from the teeth were estimated chemically.

At pH levels 2.0 and 4.0, the range in values of the dissolved Ca/P ratio is fairly small, but at pH 5.0 the range extends from 0.38 to 7.00. The possible effect of the carbonate and excess phosphate of the enamel, on the dissolved Ca/P ratio is discussed, and it is concluded that, at pH 5.0, high dissolved Ca/P ratios are associated with high calcium solubilities, and low Ca/P ratios with low calcium solubilities.

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Dietary Fluoride Related to Fluoride Content of Teeth

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Storage of fluorine in hard tissues of the human body is recognized as one of the phenomena associated with the metabolism of this element. Long-term consumption of water containing 1 ppm fluoride has been shown by analysis to increase the fluoride content of tooth structure. Dietary fluoride levels are probably related to amounts found in teeth as well, but, except for a report from Tristan da Cunha[1] suggesting this, there is no direct proof. The Canadian province of Newfoundland, like Tristan da Cunha, is insular, and its inhabitants are accustomed to a diet high in fish and tea, both of which could contribute appreciable amounts of fluoride to the daily intake. Fortunately, a number of surveys and reports concerning dietary conditions and habits in this province have been made.[2-6] The availability of such dietary information and of tooth samples for analysis from Newfoundland permitted the study reported here.

Data determined by analysis of similar teeth from the central Ontario cities of Toronto and Sarnia were also available[7] and so could serve as a control representing exposure to a low-fluoride diet in comparisons with the Newfoundland teeth exposed to a higher-fluoride diet. The work reported was an attempt to correlate dietary fluoride level with that found in human dentin and enamel.

DIETARY INFORMATION

In Newfoundland the flour consumed contributes to dietary fluoride. Since 1947 it has contained a calcium supplement of 0.5 per cent bone meal, which supplies fluoride to the diet in addition to that derived from tea and fish. This is of added importance because the nutritional surveys reported consumption of large amounts of bread and pastry in Newfoundland due to economic conditions and dietary habits.*

The diet of the large majority of Newfoundlanders at the time of the surveys (1945–48) was necessarily unvaried because of limited local production and transportation difficulties. This allowed Adamson *et al.*[2] and Cuthbertson[8] to estimate the average daily consumption of various foodstuffs available in Newfoundland. From data of McClure[9] and others the approximate intake of fluoride from these sources can be calculated (Table 1) and appears to be about 1.74 mg. per day.

The reports from Newfoundland also indicate that 1–2 cups of tea per meal are consumed even by very young children. Ham and Smith,[10] in their balance studies on young women, showed that the addition of 6–8 cups of tea per day to a normal diet in-

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* Total dietary carbohydrate 67 per cent, as compared with 51 per cent in United States and Canada (see Ref. 5).

creased fluoride intake by 0.9–1.2 mg. per day. Forty-six per cent of such ingested fluoride was retained, suggesting that tea supplies appreciable fluoride for absorption into bones and teeth. If an average of 6 cups of tea per day is consumed in Newfoundland, the daily fluoride intake would be increased to approximately 2.74 mg. fluoride per day.

The usual diet of central Ontario supplies about 0.25–0.50 mg. fluoride per day, which is an average figure as determined from studies listed in Table 2. Occasionally the daily intake would rise above this level when high-fluoride foods such as fish, spinach, or tea are consumed; on some days intake would be considerably lower. It is

TABLE 1

AVERAGE DAILY INTAKE OF NUTRIENTS IN NEWFOUNDLAND (ADAMSON *et al.* [2])

Food	Oz/day	Gm.	Ppm F	Mg. F
Dairy	5.5	150	0.09	0.013
Meat (boneless)	2.0	56	1.00	0.056
Fish (cod)	3.1	87	7.00	0.609
Eggs	0.2	6	0.60	0.004
Fats and oils	1.8			
Sugars and syrups	3.9			
Potatoes	12.3	344	0.04	0.014
Grain products	1.7	48	1.00	0.048
White flour*	12.0	336	3.00	1.00
Ingestion fluoride per day without tea				1.74
Total ingestion including 6 cups of tea per day				2.74

* Flour enriched with 0.5 per cent bone meal. Depending on the sample, the fluoride content appears to vary from 2 to 4 ppm.

TABLE 2

AVERAGE DAILY FLUORIDE INTAKE FROM NORMAL DIET

Sources of Information	Mg. Fluoride Ingested per Day
Armstrong and Knowlton (1942) (11)	0.27–0.32
McClure (1949) (12)	.25– .55
Ham and Smith (1950) (13)	.18– .30*
Ham and Smith (1954) (10)	0.43–0.73†

* Assay of typical restaurant meals in Toronto. No tea.

† Assay of meals used in balance studies of three young women in Toronto.

not likely that much more than 1 mg. fluoride per day would be ingested by individuals in Ontario, even if tea at each meal is included in the total. Neither Toronto nor Sarnia, communities compared with Newfoundland in this study, have appreciable fluoride in their respective water supplies.

Comparison of dietary fluoride intake in the two areas shows that people of Newfoundland would apparently get twice as much fluoride in the diet as those in central Ontario.

MATERIALS AND METHODS

Forty-two teeth (34 first molars, 3 second molars, 4 first bicuspids, and 1 second bicuspid) were secured from St. John's, Newfoundland, an area with no fluoride in the water supply.[14] These teeth, extracted from children between the ages of eight and

seventeen because of severe caries, had been exposed to the flour additive, bone meal, for 8 years and to the other fluoride sources for longer periods of time.

The teeth were selected at random, cleaned, ground to powder in a Wiley mill, and separated into enamel and dentin by the method of Manly and Hodge.[15] Samples were ashed, and distillation from perchloric acid was carried out following the official method of the Association of Official Agricultural Chemists.[16] Final assay of the distillates was done by Megregian's method,[17] using a Beckman DU spectrophotometer.

The ppm fluoride value determined for enamel and dentin of each tooth is plotted in Figures 1 and 2, respectively (ppm fluoride versus age of child in months). Linear re-

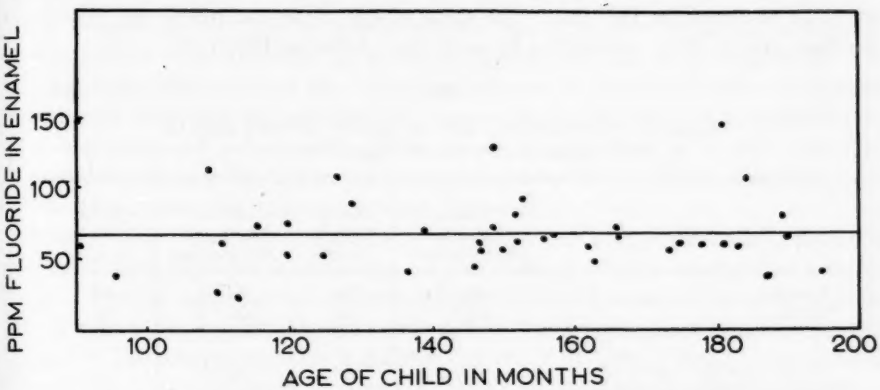

FIG. 1.—Ppm fluoride in enamel versus age of child

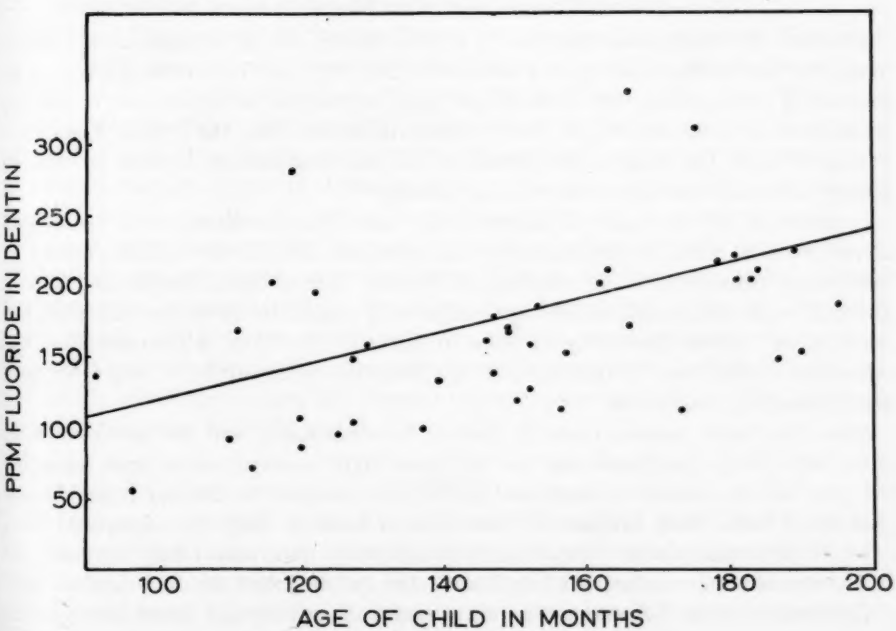

FIG. 2.—Ppm. fluoride in dentin versus age of child

gression equations were calculated for each set of data (enamel and dentin), and the slopes (linear regression coefficients) obtained were tested for significance by comparison with zero in a t-test—$t = (b - 0)/\text{s.e. } b$.* The regression lines were also plotted in the figures.

RESULTS

Enamel.—The linear regression equation ($y = 67 + 0.13x$; s.e. of estimate = 25) showed a very slight slope of 0.13 ± 0.14 s.e., representing increase in fluoride content of enamel per month. A t-test of the significance of this increase gave a value of $t = 0.93$ (38 D.F.), indicating a probability of about 25 per cent that a slope of zero would be observed for this data. The slope of the regression line is not significant, therefore, and so it was assumed to be zero when plotted in Figure 1.

TABLE 3

AVERAGE FLUORIDE CONTENT OF DENTIN AND ENAMEL OF CHILDREN'S TEETH (AGE 8–17)

City	Enamel (Mean±s.e. Ppm Fluoride)	No. of Teeth in Sample	Dentin (Mean±s.e. Ppm Fluoride)	No. of Teeth in Sample
Toronto	46±3	66	91±6	69
Sarnia	48±7	22	104±9	24
St. John's (Newfoundland)	67±4	40	164±9	42
Toronto-Sarnia (combined)	47±3	88	94±5	93
Stratford (1.3 ppm fluoride in water supply)	128±16	11	325±28	11

Dentin.—The regression equation ($y = 23 + 0.95x$; s.e. of estimate = 55) determined for the dentin values gave a slope of 0.95 ± 0.31 s.e. This value represents an increase of 11.4 ppm fluoride in dentin per year. A t-test of the significance of this increase gave a value of 3.09 (40 D.F.), which is greater than the critical 1 per cent t-value of 2.71. The slope of the regression line, showing average increase in fluoride content of dentin with age, is therefore significant.

Comparison of averages (Toronto-Sarnia and Newfoundland).—As mentioned above, previous work in this laboratory has provided data on the fluoride content of dentin and enamel from various districts in Ontario.[7] Two of these, Toronto and Sarnia, although separated geographically, are similar with respect to the dietary habits of the populace and lack of fluoride in the water. A third city, Stratford, while resembling the other cities in diet, has 1.3 ppm natural fluoride in the water supply.[13] Data from here are reported for comparison.

Since the tooth samples from St. John's, Newfoundland, and the above Ontario cities were evenly distributed over the age range eight to seventeen, a mean value for the ppm fluoride content of dentin and enamel was calulated for this age interval from each set of data. These averages are presented in Table 3. They were compared by a t-test to determine whether they differed significantly from each other, Toronto and Sarnia representing exposure to a low-fluoride diet and St. John's to a high-fluoride diet.

Comparison of the Toronto and Sarnia averages for enamel and dentin showed that

* s.e. = standard error; b = linear regression coefficient.

they did not differ significantly from each other, and so these two groups of Ontario data were combined for comparison with the Newfoundland data.

Comparison of the means of Newfoundland and Toronto-Sarnia enamels gave a *t*-value of 3.37 (120 D.F.), significant at the 1 per cent level. Comparison of the dentin averages of these two gave a *t*-value of 6.94 (133 D.F.), again highly significant. It may be concluded, therefore, that the teeth of St. John's, Newfoundland, children contain significantly more fluoride than do those of Toronto-Sarnia children of the same age.

DISCUSSION

The findings for St. John's, Newfoundland, enamel suggest either no increase in fluoride content after a certain age or an increase too gradual to be measurable. The fact that little replacement of the constituents of enamel takes place after calcification and eruption of a tooth suggests that the observed deposition of fluoride occurred during calcification and subsequently either ceased or progressed at a very slow rate. Absorption of dietary fluoride on the enamel surface of the teeth may also take place, but the data cannot give any measure of its importance.

Examination of the St. John's dentin results shows that the ppm fluoride in dentin of children's teeth increases with the age of the child. A definite relationship between levels of fluoride occurring in the diet and amounts found in dentin is therefore probable. A similar relationship has been observed for water-borne fluoride and fluoride in dentin.[7, 18] The average increase in fluoride content of St. John's dentin is found to be 114 ppm for a 10-year period. It would be interesting to determine whether the rate of accumulation of fluoride in dentin is maintained during the life of the individual. A study to investigate this is at present under way in our laboratory.

The fluoride responsible for the significantly greater amounts observed in Newfoundland teeth as compared with Toronto-Sarnia teeth must have been absorbed from high-fluoride foods in the Newfoundland diet (tea, fish, and bone meal), since there is no other source. The diet in Toronto-Sarnia does not produce the fluoride level observed in St. John's teeth. This finding suggests that in planning a program of public water fluoridation the dietary habits of the populace may have to be considered so as to avoid the slight excesses of fluoride that can cause some individuals to develop fluorosis.

Table 3 shows that Stratford averages for fluoride content of dentin and enamel resulting from consumption of water-borne fluoride are much higher than those produced by ingestion of dietary fluoride in St. John's. It is known that 1 ppm fluoride in the water supply will decrease caries incidence, and this amount caused the levels of fluoride observed in Stratford dentin and enamel. It would seem that the fluoride content of St. John's teeth is not high enough to have any caries-reducing effect. Investigation of this point might be useful.

SUMMARY

1. Each of 42 permanent teeth extracted from children in St. John's, Newfoundland, was separated into enamel and dentin, and the two fractions were analyzed for fluoride. The analytical results were compared statistically with results from similar assays of teeth from Ontario cities. The St. John's teeth were exposed to a high-fluoride diet, while those from Ontario represent exposure to a low-fluoride diet.

2. It is concluded that some of the fluoride present in the foods—tea, fish, and bone meal—can be absorbed and retained in human teeth.

3. Evidence presented indicates that the use of these high-fluoride-content foods in the diet leads to an increased level of fluoride in the dentin and enamel of children's teeth in St. John's, as compared with teeth from children on the low-fluoride Ontario diet.

4. The amount of fluoride in the dentin of children's teeth in St. John's is found to increase with the age of the child.

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The Isolation of Certain Types of Micrococci from Dental Students and Related Antibiotic Studies

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The recent rise in the incidence of antibiotic-resistant strains of pathogenic micrococci and in the percentage of surgical postoperative infections attributable to these organisms suggests the need for a specific investigation of this field as it relates to dentistry.[1-6]

Many implications have arisen in considering the treatment of postoperative micrococcal oral infections resulting from dental surgery. With reports from Benham *et al.* and Landy *et al.*, the epidemiological concept of oral infections has changed greatly; the thought that the mucosal membranes of the surgeon himself might be the source of the infective agent had not been widespread.[7, 8]

No work has been reported interrelating the dentist and patient to the transfer of etiologic agents in postoperative infections. Considering the proximity of dentist and patient in the normal course of operative and surgical proceedings, an investigation into this area should prove most valuable in determining the course of such microbiologic transfer and resulting infections.

Basing their work on preliminary studies, the authors have selected the following objectives for consideration: (1) the incidence of micrococcal organisms in the nasal mucosa, with special emphasis on those pathogens demonstrating hemolytic and serum coagulase activity; (2) a study of antibiotic-resistance patterns of isolates; and (3) studies of acquired resistance. Experimental results obtained by utilizing these objectives as a guide should provide some insight into the problem of postoperative infection.

HISTORICAL REVIEW

Since the initiation of antibiotic therapy subsequent to the discovery of penicillin, micrococcal resistance patterns have changed markedly and swiftly. Increased antibiotic resistance of all micrococci has presented a serious surgical problem to the general surgeon and dental surgeon alike.

The penicillin-resistant pathogenic micrococci encountered in hospital laboratories have mounted steadily since 1943. Spink reported only 12 per cent of all cultures encountered as resistant.[9] Gallardo confirmed this.[10] Blair and Barber and Dorozenko reported steadily increasing percentages of penicillin-resistant strains of 21.5, 41.0, and 59, respectively, for the years 1945, 1946, and 1948.[11, 12] More recently Nichols and Needham in 1949 and 1953, Biegelman and Rantz in 1950, and Tamblyn and

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Moyer in 1954 reported, respectively, 68, 56, and 82 per cent of hospital isolates as being penicillin-resistant in the years of publication listed.[6, 13-15]

Based on the severe problem presented by postoperative micrococcal infection, Benham *et al.* studied antibiotic sensitivity patterns of *Micrococcus pyogenes* from infected surgical wounds and compared these with patterns of those organisms isolated from the respiratory tracts of surgery personnel.[7] In vitro sensitivity assays of penicillin, chlortetracycline, oxytetracycline, chloramphenicol, bacitracin, and streptomycin were studied. Of the strains isolated from surgical wounds following treatment with penicillin and streptomycin, 94 per cent were resistant to penicillin, and 79 per cent were resistant to streptomycin *in vitro*. Of the strains isolated from surgery personnel, 19 per cent were sensitive to penicillin and 81 per cent resistant. The predominant pattern among strains isolated from wounds indicated resistance to penicillin, streptomycin, chlortetracycline, oxytetracycline, and tetracycline. The general pattern (36 per cent) among surgery personnel was one of resistance to penicillin only. Where infection of surgical wounds with *M. pyogenes* occurred, a definite correlation between the sensitivity of the organisms recovered from such wounds and that of those from respiratory tracts of surgery personnel was indicated.

Further investigations in the field, opened by the work of Benham *et al.*,[7] led researchers at the University of Chicago under Landy to study surgical wound infections in seven clinical cases occurring within 13 days.[8] These micrococcal infections were traced to contamination by organisms from the nasal mucosa of the first assistant surgeon. The etiologic agent was described as "positive, plasma clumping" *M. pyogenes* var. *aureus*. This organism displayed the following antibiotic sensitivity pattern: resistant to penicillin, streptomycin, aureomycin, terramycin, and tetracycline; sensitive to chloramephenicol, bacitracin, erythromycin, and magnamycin. All seven wounds demonstrated the same organism with identical sensitivity patterns. Direct correlation of the antibiotic tube-dilution assay with the impregnated-disk method was definitely established through comparative studies. Control cultures of the nasal mucosa of patients, surgeon, and second assistant disclosed no pathogenic micrococci. Proof of patient-operator transfer of etiologic agent was demonstrated by the occurrence of fewer infections when the first assistant surgeon donned two masks and extended the scrubbing time. However, this first assistant retained identically resistant micrococci, described above, as normal nasal flora for 2 years. This work established the pattern for the etiology and epidemiology of many postoperative infections.

Numerous and conflicting results have been reported concerning the resistance of various organisms toward many of the antibiotics. Baron reported no increased resistance by *M. pyogenes* var. *aureus* toward aureomycin when cultured with increasing concentrations of the antibiotic.[16] Lawrence and Francis have described the development of drug resistance to aureomycin to be "infrequent and generally of a low degree."[17] Pratt and Dufrenoy also report the development of bacterial resistance to erythromycin.[18] Lubash, Van der Meulen, Berntsen, and Tompsett have reported on the production of a strain of *M. pyogenes* var. *aureus* resistant to novobiocin.[19] In a similar manner Jones, Nichols, and Finland have noted that "the rate of development of resistance in strains of *M. aureus* during repeated subcultures on antibiotic containing agar was rapid."[20]

Basic knowledge, provided by a variety of investigators, as to the swift increases in

antibiotic-resistant pathogenic micrococci over a period of years, coupled with recent correlation of surgeon-to-patient transmission of etiologic agents, has indicated the importance of a greater knowledge of the distribution and activity of micrococcal flora of the dentist, dental surgeon, and patient. The proximity of dentist and patient in operative procedures has prompted the desire for further investigation into the pathogenic micrococcal flora of the nasal mucosa of dental students, in an attempt to survey both the distribution of the organisms mentioned above and the antibiotic sensitivity patterns of such organisms.

A discrete knowledge of the pathogenic micrococcal population of the nasal mucosa of dental students and antibiotic resistance patterns of this group may further aid the application of postoperative therapy. Clarification of dentist-patient transfer of an etiologic agent may result from studies directed along these lines. Investigations into the antibiotic resistance patterns and resistance acquisition, as modified by mutational variation, are reported with the hope of providing a better understanding of the phenomenon and treatment of postoperative infection.

MATERIALS AND METHODS

Cultures of micrococci were isolated from nasal swabbings of 347 dental students and were maintained on brain-heart infusion agar slants. All cultures were collected during the months of March and December.

All media and glassware, except the dehydrated human plasma (supplied sterile), were sterilized by autoclaving; antimicrobial "sensi-discs"* for sensitivity tests were supplied sterile, and whole human blood was supplied from a hospital blood bank.

Specimens were obtained by introducing sterile cotton swabs, moistened with 0.85 NaCl solution, $\frac{1}{2}$ inch into the inferior meatus of the nose. The specimens were streaked directly onto blood agar and incubated at 37° C. for 24 hours. Micrococcal strains showing beta hemolysis were transferred to brain-heart infusion agar slants for subsequent analysis. Coagulase activity tests were performed using desiccated plasma.[21] Only organisms demonstrating beta hemolytic action and coagulase activity were considered as pathogens.[22,23] As an adjunct for further separation of pathogenic from non-pathogenic micrococci mannitol fermentation tests were performed on the isolates.[24]

Sensitivity tests were performed on trypticase agar, utilizing the "sensi-disc" technique. Two concentrations of each antibiotic were used. No attempt to correlate zone size of different antibiotics was attempted, owing to unknown variation in the rates of diffusion and efficiency of antibiotics in vitro.[25]

To determine the rapidity with which these micrococcal isolates become drug-tolerant, certain isolates were selected to be subjected to an environment containing increasing concentrations of certain antibiotics. Nine isolates were selected on the basis of a marked susceptibility toward aureomycin, erythromycin, and novobiocin. Seven of the nine strains were pathogenic is determined by their ability to ferment mannitol, clot normal human plasma, and produce zones of hemolysis on blood agar plates. The remaining tow strains were non-pathogenic.

A tube-culture titration was performed to ascertain the baseline tolerance of each organism toward each antibiotic. By consecutive culturing of the organisms in increas-

* Sensi-disc," registered trade mark, Baltimore Biological Laboratory, Baltimore, Maryland.

ing concentrations of each antibiotic, an attempt was made to raise the level of tolerance of each organism to the individual antibiotics. Ten consecutive transfers were made over a period of 12 calendar days. The incubation increments ranged from 24 to 72 hours, depending upon the time required to produce visible turbidity in the culture tube.

Crystalline aureomycin HCl,* erythromycin glucoheptonate,† and novobiocin,‡ were the antibiotics utilized.

The tube-dilution method was utilized to obtain the desired concentrations of the antibiotics.

RESULTS AND DISCUSSION

Patient history.—A survey of the patient's general health was conducted, primarily to determine the therapeutic usage of antibiotics by the patient in recent years.

Besides revealing factors influencing the sensitivities of the nasal flora of the individual, the history also provides basic information concerning the patient's general health and past micrococcal infections. The completed chart gives a concise record of the historical and experimental data. Nasal swabs were taken from 347 dental students. The incidence of micrococci isolated from such swabbings was 98 per cent.

The incidence of pathogenic micrococci for the four groups was 23.5 per cent. The incidence per group varied from 12 to 34 per cent.

No apparent pattern was established for numbers of non-pathogenic or pathogenic micrococci between the various clinical and preclinical groups.

The majority of microörganisms recovered were of the *M. pyogenes* var. *albus* type, while the majority of pathogens recovered were of the *M. pyogenes* var. *aureus* type.

It was routine to isolate two or more different morphologic and hemolytic types of micrococci from a single swabbing (Table 1).

Antibiotic resistance patterns.—Organisms when subjected to the activity of aureomycin, carbomycin, chloromycetin, neomycin, streptomycin, matromycin, novobiocin, terramycin, tetracycline, and erythromycin demonstrated little or no resistance to the concentrations utilized. Strains tested in vitro against polymyxin showed a general resistance to the antibiotic. Only in the case of penicillin was a definite pattern established. Resistance was apparent in approximately 33 per cent of all strains tested. Penicillin-susceptible strains showed extreme sensitivity to high and low concentrations of the drug.

Four *M. pyogenes* var. *aureus* strains that demonstrated no coagulase activity were selected for sensitivity studies, in speculation that some detectable relationship between coagulase activity and antibiotic resistance might be demonstrated. Results indicated that the general pattern of the coagulase-negative strains shows a greater degree of resistance to many of the antibiotics than those patterns of the coagulase-positive strains.

Acquired resistance studies.—Table 2 discloses the variations in antibiotic tolerance. It is interesting to note that there is no apparent difference between the non-pathogens (organisms H and I) and the pathogens in regard to their ability to become drug-tolerant.

* Lederle Laboratories Division, American Cyanamid Company.

† Eli Lilly and Company. ‡ The Upjohn Company.

The experimental results demonstrate, to varying degrees, the ability of each of these nine isolates to become more tolerant toward certain antibiotics. The ratio of initial to final concentration varied from 2 to 82 with aureomycin, 3 to 115 with novobiocin, and 0 to 240 with erythromycin. The figures thus obtained with aureomycin are inconsistent with those reported by Baron, who found no increased resistance in the ratio of initial to final concentration of aureomycin after fourteen transfers on media containing increasing concentrations of aureomycin.[16] These data, however, are in accord with work reported by Lubash, Van der Meulen, Bernsten, and Tompsett, as well as by Jones, Nichols, and Finland.[19,20]

The results obtained from this study serve to illustrate that the acquisition of drug

TABLE 1

HEMOLYTIC CHARACTER, COAGULASE ACTIVITY, AND ACTION IN 5 PER CENT MANNITOL BROTH OF MICROCOCCAL ISOLATES

DESCRIPTION		GROUP					
		Ip*	IIp	Total	IIc†	IIIc	Total
No. of swabs taken		73	104	177	101	69	170
Cultures demonstrating micrococci		73	102	175	98	67	165
No. hemolytic micrococci	*M. aureus*	16‡	31	47	33	9	42
	M. albus	56	77	133	96	57	153
	Total	72	108	180	129	66	195
No. hemolytic, coagulase-positive micrococci	*M. aureus*	15	27	42	33	7	40
	M. albus	2	1	3	0	2	2
	Total	17	28	45	33	9	42
No. coagulase-positive, hemolytic, manitol-fermenting micrococci	*M. aureus*	13	23	36	33	7	40
	M. albus	2	1	3	0	1	1
	Total	15	24	39	33	8	41

* p = preclinical students.
† c = clinical students.
‡ Figures represent total strains exhibiting trait indicated.

TABLE 2

INCREASE IN ANTIBIOTIC TOLERANCE OF SELECTED MICROCOCCI

CULTURE	AUREOMYCIN			NOVOBIOCIN			ERYTHROMYCIN		
	Min.	Max.	Fold Increase	Min.	Max.	Fold Increase	Min.	Max.	Fold Increase
A	0.15*	7.3	50†	0.15	8.0	5	0.025	0.05	2
B	.15	1.7	10	.1	7.4	74	.2	4.2	21
C	.25	0.7	3	.1	8.4	84	.2	2.5	12
D	.15	0.35	2	.15	2.1	14	.1	3.5	35
E	.15	6.9	5	.15	7.7	51	.1	5.0	50
F	.15	1.0	7	.1	11.5	115	.2	5.2	26
G	.15	0.5	3	.5	8.2	16	.2	0.25	0
H	.1	8.2	82	.5	1.5	3	.025	6.0	240
I	0.2	10.8	50	0.15	6.2	41	0.15	0.3	2

* Minimum and maximum figures represent micrograms of antibiotic per milliliter of broth culture.
† Fold-increase figures are approximated.

tolerance by micrococci is fairly rapid and that the levels of tolerance achieved, within the limits of this experiment, can be multifold.

SUMMARY AND CONCLUSIONS

An investigation into the micrococcal flora of the nasal mucosa of 347 dental students is presented.

No significant pattern was established in the recovery of pathogenic micrococci isolated from preclinical and clinical students. The percentage recovery of micrococci in all groups approached 100, while 23.5 per cent of this total represented pathogens.

All pathogenic isolates were subjected to sensitivity testing with a variety of proprietary antibiotics. The organisms demonstrated little or no resistance to the majority of drugs tested. There was a general pattern of resistance to the action of polymyxin. Only extremes were noted with penicillin—organisms being generally sensitive or resistant to the concentrations of antibiotic utilized.

The possible interrelationships of acquired antibiotic resistance and the importance of the determination of micrococcal populations in the treatment of postoperative infection are presented. Multifold increases in antibiotic tolerance and the rapid rate of the acquisition of such tolerances are reported for selected micrococci.

The import of greater understanding of the phenomenon of postoperative infection as related to dentist-patient proximity under operative and surgical technics is implicated.

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A Comparison of the Harvard 700 Diet (Modified) with the HWC Diet (Modified) for Inducing Occlusal Caries in Resistant and Susceptible Rats

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The development of caries-resistant and caries-susceptible rats by Hunt and Hoppert[1–6] was facilitated by the Hoppert-Webber-Canniff (HWC) coarse-particle diet.[7,8] The difference between the two lines was still evident when certain high-sucrose diets were used.[9,10] Shaw reported that a high-sucrose ration caused greater destruction of the teeth than did the HWC diet.[11] This investigation was designed to assess the cariogenicity of this diet and to determine whether the difference between the two strains persisted. Lactobacilli and streptococci were quantitatively determined, in order to ascertain the relationship between diet, caries, and these microörganisms.

EXPERIMENTAL METHODS

The rats used in this experiment were from the twenty-seventh generation of the resistant line and the second and third generations of the OMS* line. A non-cariogenic diet† was supplied from a few days before birth of the rats to about 35 days of age. Littermates of each line were divided into three groups; each group was fed one of the following cariogenic diets: (1) a modified HWC coarse-particle diet;[12] (2) the Harvard high-sucrose 700 diet + 15 per cent celluflour, hereby designated as "700+";[13,14] and (3) the HWC diet plus the vitaminized casein of the 700+ diet and hereby designated as "HWCV." The third diet was used because it was believed that the addition of free vitamins might stimulate the growth of cariogenic microörganisms.

Caries was scored by determining the time in days that it took a rat to develop a lesion that could be seen with the naked eye in the living aimal. Only gross occlusal fissure caries was looked for, and other types of carious lesions were not taken into consideration. Details of this procedure have been presented previously.[5,12]

Oral lactobacilli and streptococci were determined by a swab culture technique. Rogosa SL agar and CVA medium, respectively, were used to determine the numbers

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* The reproduction of the caries-susceptible line was so poor in recent years that fertile susceptible rats were crossed with Osborne-Mendel stock obtained from the National Institutes of Health. The new line, called Osborne-Mendel-Susceptible (OMS), is fecund, vigorous, and uniformly susceptible.

† This diet consists of whole powdered milk, 74 per cent; casein, 10 per cent; dried brewer's yeast, 5 per cent; alfalfa, 10 per cent; and sodium chloride, 1 per cent.

of these two groups of microörganisms. The detailed bacteriologic methods were presented in a previous publication.[12]

RESULTS

The data in Table 1 show a distinct difference between caries-resistant and OMS rats when either a coarse-particle or a high-sucrose diet was used. It took 217.0 ± 16.8 days for the resistant rats to develop caries on the HWC coarse-particle diet, whereas it required only 49.0 ± 3.4 days for OMS rats to develop caries on the same diet. On the high-sucrose diet, the resistant rats developed caries in 400.4 ± 21.6 days, but the OMS rats developed caries in only 122.4 ± 13.9 days. It took considerably longer for rats to develop caries on the sugar diet than on either of the coarse-particle diets

TABLE 1

CARIOGENICITY OF HIGH-SUCROSE DIET (700+) AND COARSE-PARTICLE DIETS (HWCV AND HWC)

Diet	Strain of Rats	No. of Rats	Caries Time (Days) and Standard Error	*P*
700+	Resistant	20	400.4±21.6	<0.01
HWCV	Resistant	18	244.3±19.5	≥0.3
HWC	Resistant	19	217.0±16.8	<0.01
700+	OMS	18	122.4±13.9	<0.01
HWCV	OMS	15	48.8± 5.7	0.05
HWC	OMS	16	49.0± 3.4	<0.01

($P<0.01$). The addition of the vitaminized casein of the 700+ diet to the HWC ration (HWCV) did not appreciably affect the development of caries ($P \geq 0.3$). That is, the two coarse-particle diets (HWCV and HWC), with or without free vitamins added, produced caries in about the same period of time.

The numbers of microörganisms recovered are presented in Figure 1. The numbers of rats used for each experimental group are given in Table 1. Lactobacilli were recovered in higher numbers from OMS than from caries-resistant rats when given a coarse-particle diet (HWC or HWCV). This finding confirms previous reports.[15-17] However, the numbers of lactobacilli recovered from rats on the high-sucrose diet (700+) was about the same for both strains of rats. Therefore, the association of lactobacilli with dental caries was evident only when rats were fed a coarse-particle diet.

There was a greater degree of association of streptococci with caries than of lactobacilli with caries. All the OMS rats had high populations of streptococci in both examinations. After 6 weeks on the experimental diet, the streptococcal count was low

in the resistant rats but was much higher after 23 weeks. By this time, a few of the resistant rats had already developed caries, and probably caries was being initiated in many more resistant rats.

DISCUSSION

Although the Hunt-Hoppert caries-resistant and caries-susceptible lines were developed with a coarse-particle diet, the difference between the two lines was still great when a high-sucrose diet was used.[9,10] There was also a wide difference in caries ex-

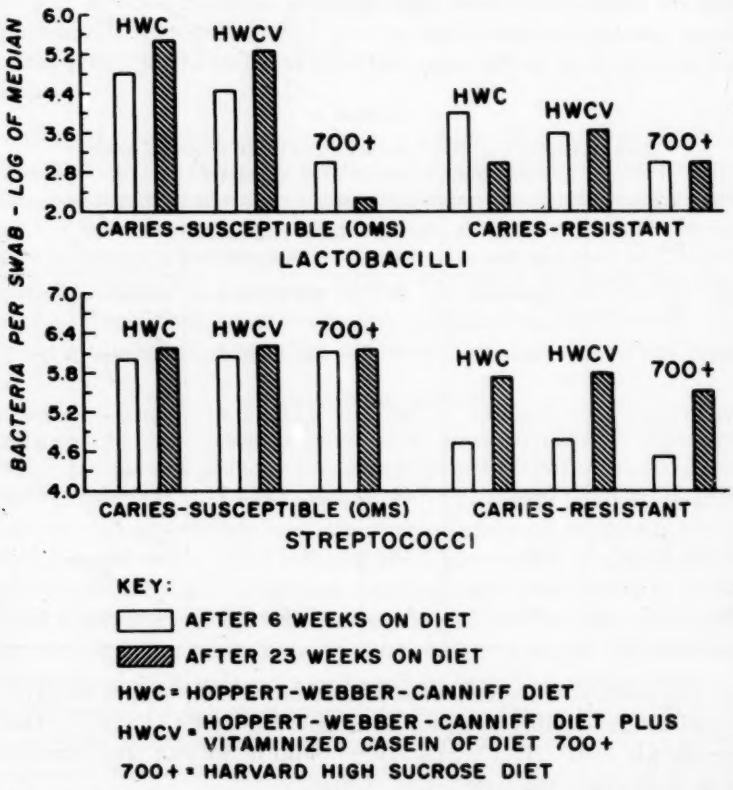

FIG. 1.—Populations of lactobacilli and streptococci in response to several cariogenic diets

perience between the Hunt-Hoppert caries-resistant rats and OMS rats when the modified Harvard 700 diet was used. The possibility exists that certain cariogenic diets may not bring out a distinct difference between Hunt-Hoppert caries-resistant and OMS rats. Until this is established, the results to date suggest a "common denominator" for susceptibility to caries. That is, genes responsible for susceptibility establish favorable oral environmental conditions for the development of caries in susceptible rats regardless of the nature of the cariogenic diet.

The rate at which caries developed in rats fed a high-sucrose diet was lower than in rats fed a coarse-particle diet. The lactobacillus count was much higher in the groups

of rats given coarse-particle rations. This suggests that lactobacilli enhanced the development of caries. The coarse-particle ration favors the growth of lactobacilli probably by allowing for greater food accumulation in the fissures of the rats' teeth.

SUMMARY

The difference between caries-resistant and OMS rats developed by Hunt and Hoppert was evident when either a coarse-particle or a high-sucrose diet was used. Both OMS and resistant rats developed caries faster on the coarse-particle diet than on the high-sucrose diet.

Lactobacilli were recovered in higher numbers from OMS than from caries-resistant rats only when the coarse-particle diet was used. Streptococci were recovered in higher numbers from OMS rats when either the coarse-particle or the high-sucrose diet was used.

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An Improved Technique for the Paper Electrophoresis of Parotid Saliva

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Paper electrophoresis is an effective method for the separation of mixtures of charged macromolecules in solution and has proved particularly valuable for analyses of blood serum. However, application to studies on saliva has been limited. Kinersly[1] and Kinersly and Leite[2], Verschure,[3] and Gabl and Egger[4] worked with whole saliva of human beings but did not report as extensive separations as one might expect from a fluid known to contain a complex of components. Zeldow[5] followed the techniques of Kinersly[1] in studying human parotid saliva, a somewhat less complex secretion, and was able to show two zones. The knowledge obtained by these workers suggested that it might be possible to delineate more components in human parotid saliva if the technique was modified specifically for this secretion. Recently, Ferguson, Kraun, and Hildes,[6] Drevon and Donikian,[7] and Mandel and Ellison,[8] with different degrees of success, have been able to separate human parotid saliva into zones varying from two to nine in number, and Hoerman[9] has reported the separation of components in parotid saliva by the use of zone electrophoresis in starch gels.

There was little doubt that several more components existed in saliva, or parotid saliva, since these were known from studies using the classical method of Tiselius.[10–17] However, for clinical studies, paper electrophoresis offers a much simpler and yet definitive procedure. Moreover, parotid saliva is more easily obtained and tends to be less complicated than whole saliva because of the absence of food debris and the low numbers of bacteria and tissue cells. Thus the purpose of this report is to describe a reproducible method for clear separation of components in human parotid saliva.

MATERIALS AND METHODS

Collection.—Parotid saliva was collected by means of a modified Curby cup,[18] which was attached over the orifice of Stenson's duct after the buccal mucosa was treated with two applications of an iodine antiseptic. The saliva-collecting tube was immersed in an iced vessel, in order to minimize protein alterations and enzymatic activity. Salivary flow was increased by the use of flavored troches.

Dialysis.—Each 5-ml. saliva sample was dialyzed against 1,500 ml. distilled water at 5° C. overnight to remove salts. Crystalline barium chloride and silver nitrate were

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added to aliquots of the dialysate sample to detect the presence of inorganic salts. Precipitate in any test indicated the necessity for additional dialysis.

Concentration.—The organic components in parotid saliva are in such low concentration[19, 20] that separation is difficult to visualize (after staining) without concentration of the sample. This concentration was accomplished by dividing each sample into portions containing an equivalent of 3 ml. of parotid saliva, placing them in sterile 30-ml. vaccine bottles, and lyophilizing for 12 hours. When used, an aliquot was reconstituted with 0.15 ml. of veronal buffer, ionic strength 0.05 and pH 8.6. Thus each sample during electrophoresis contained twenty times more organic material per unit volume than did the original sample. Thirty minutes at 21° C. were allowed for the mixture to dissolve. Usually, very little or no sediment was visible in the dissolved sample, but no attempt was made to remove any sediment.

Apparatus.—The electrophoresis apparatus used was a Spinco Model R Cell (Series C).

Strip preparation.—Paper wicks were inserted into the cell, and each paper strip, Schleicher and Schuell No. 2043A, was lightly marked at the center to identify the line of application. The strips were then placed on the rack with tweezers, transferred to the cell, and moistened by adding 5 ml. of buffer to each strip. The remainder of the buffer solution was added directly to the cell. After 15 minutes, the lucite cover was replaced, and the cell was permitted to remain with the unsealed lid in place for an additional 15-minute period, a total time of 30 minutes having elapsed. These latter details in the procedure are important, for they permitted the strips prior to the application of the sample to absorb an optimal amount of moisture but did not allow them to become as highly saturated with the buffer as did previous methods described in the literature[21] and in our own laboratory.

Application, electrophoresis, and drying.—An 0.01-ml. portion of the reconstituted saliva was transferred to the applicator and was deposited evenly on the center line of each strip. The application slit was sealed with moisture-proof plastic tape and the current adjusted to supply a 200-volt difference in potential across the strips for a 23-hour period. The filtered, direct current was supplied by a rectifier power supply (dual capacitor–choke filter) which produced variable direct current (0–350 V.) rated at 100 ma. with a very low ripple. Electrophoresis was conducted at either 5° or 21° C. At the end of 23 hours the strips were left on the rack and were placed in an oven at 120° C. for 15 minutes.

Dyeing.—After drying, the strips were removed and placed in a metal dyeing frame. Several dyes were tested in an effort to determine which one would be the most effective for staining the separated components. The dye formulations and the staining procedures may be found in the reference following each dye: bromphenol blue stain (Difco);[22] amidoschwartz 10b stain (Harleco);[22] azocarmine G stain (Harleco);[22] and light-green SF stain (National Aniline).[23] After the strips were dyed, they were analyzed by gross inspection and by a densitometer, Spinco Model RB, employing suitable interference filters.

RESULTS AND DISCUSSION

The results of some 148 experiments are best presented by reviewing a series of typical electrophoretograms and their corresponding absorption curves. Figure 1, *a–d*,

show the electrophoretograms and absorption curves obtained from a simultaneous study of four separate portions of a single parotid saliva sample, each of which was run at 5° C. The only difference in the treatment of the paper strips was in the dyeing. Application of bromphenol blue[21] (Fig. 1, *a*) revealed one distinct band containing a high concentration of stainable material which migrated slowly toward the anode. A less distinct, but seemingly broad, band may also be seen in the absorption curve. This band contains much less stainable material and moved toward the cathode. The strip stained with azocarmine G[22] is shown in Figure 1, *b*. The strip stained very similarly to the one in Figure 1, *a*.

The use of light-green SF dye[23] (Fig. 1, *c*) indicated greater separation than in the

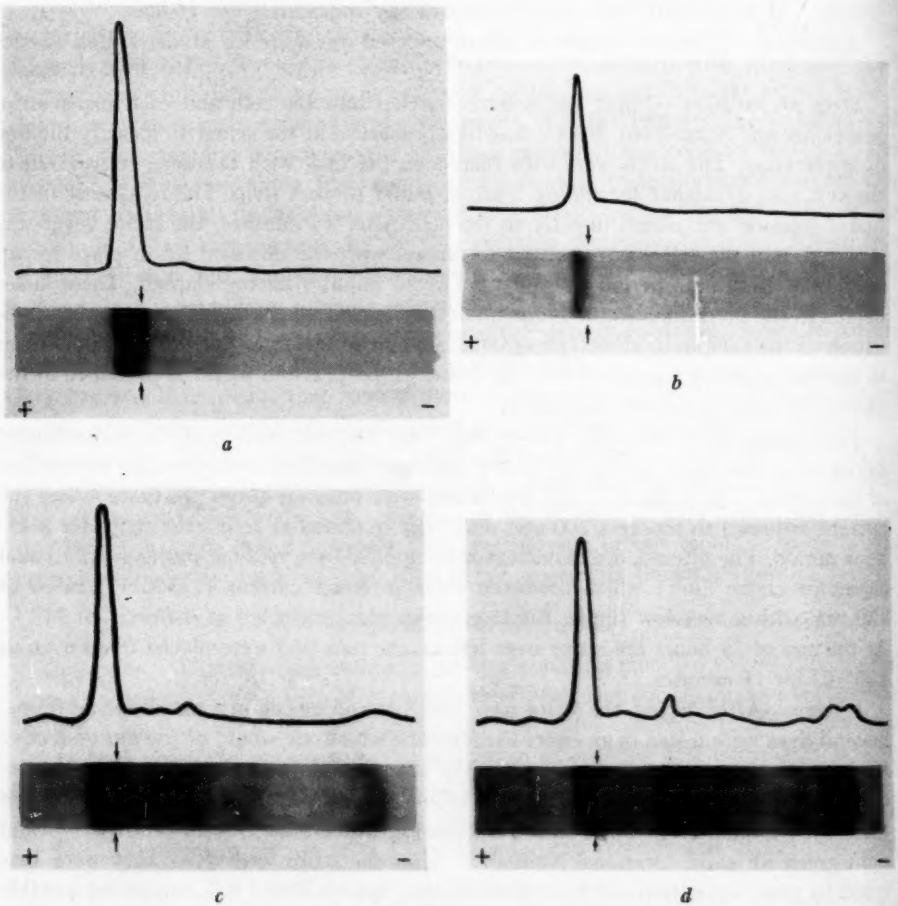

FIG. 1.—Parotid saliva, 20× conc.; 0.01-ml. sample; 23 hours; 200 VDC; 0.17 ma/cm width of filter paper; Schleicher and Schuell paper No. 2043A; veronal buffer, ionic strength 0.05, pH 8.6; 5° C.; starting lines are indicated by arrows, and the polarity of the strips is designated; strips were stained as indicated. (*a*) Bromphenol blue stain scanned with 600-mμ interference filter; (*b*) azocarmine G stain scanned with 550-mμ interference filter; (*c*) light-green SF stain scanned with 600-mμ interference filter; and (*d*) amidoschwartz 10b stain scanned with 650-mμ interference filter.

previous strips. The band containing the high concentration of stainable material was still present and migrated in the same direction. The main differences, however, were in the appearance of four faintly staining bands which migrated toward the cathode and one faint band which migrated toward the anode at a faster rate than did the heavily stained band.

Amidoschwartz 10b[22] was the most effective dye in this series. Inspection of the strip (Fig. 1, *d*) shows that there were a total of eleven components, of which eight components migrated toward the cathode and three migrated toward the anode. Of the eight components that traveled toward the cathode, three appeared to have a greater affinity for the dye than did the other components that traveled in the same direction. The component that moved the slowest toward the cathode was a lightly staining ill-defined band. It appeared only when electrophoresis was conducted at 5° C. Of the three

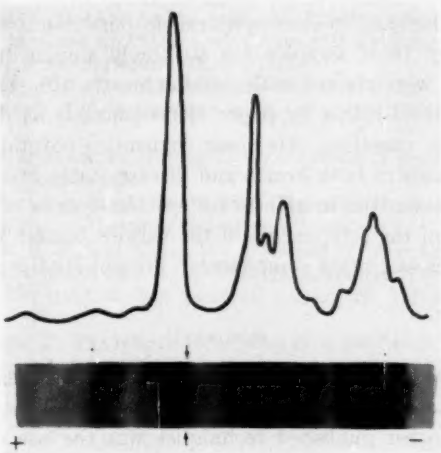

FIG. 2.—Parotid saliva, 20× conc.; 0.01-ml. sample; 23 hours; 200 VDC; 0.25 ma/cm width of filter paper; Schleicher and Schuell paper No. 2043A; veronal buffer, ionic strength 0.05, pH 8.6; 21° C.; starting line is indicated by arrows, and the polarity of the strip is designated. Amidoschwartz 10b stain scanned with 650-mμ interference filter.

components that traveled toward the anode, two were quite indistinct and had a greater mobility than the third, which was the heaviest-stained band and the band that characteristically appeared in all the previous strips (Fig. 1, *a–c*). The results of these separations carired out at 5° C. led us to believe that amidoschwartz 10b was the stain of choice under these conditions, as it revealed the largest number of components.

The series of analyses just described indicated that parotid saliva contained several components separable by paper electrophoresis. We then attempted to obtain better separation of components by changing the temperature under which electrophoresis was conducted. Figure 2 shows a typical electrophoretogram and absorption curve obtained at 21° C. and stained with the use of amidoschwartz 10b stain technique. This particular saliva sample was obtained from the same donor as in Figure 1 but was collected at a later date. Examination of the strip (Fig. 2) showed that seven components migrated toward the cathode and four migrated toward the anode. Of the seven components that migrated toward the cathode, three appeared to have the same affinity

for the dye as did those seen in Figure 1, *d*. Four components appeared which traveled toward the anode. Again one of these was a slowly moving component that had a greater affinity for the dye than did the three other lighter-staining components.

The main differences between Figure 1, *d*, and 2 were the following: (1) the over-all increase in density of the various components in Figure 2 compared to Figure 1, *d*. This is shown graphically by the increase in the height of the peaks on the corresponding absorption curves (Scardi and Bonavita).[24] (2) The sharper distinction of the components in Figure 2. (3) The absence in Figure 2 of the lightly staining hazy component that migrated at the slowest rate toward the cathode. (4) The appearance in Figure 2 of an additional lightly staining component that traveled toward the anode which was absent in Figure 1, *d*. (5) The components in Figure 1, *d*, uniformly moved more rapidly than did their counterparts in Figure 2.

Since the work described here was completed, numerous "normal" parotid saliva samples have been submitted to electrophoresis in our laboratory. The electrophoretic patterns evidenced by these samples are strikingly similar to the one pictured in Figure 2 when strips were stained with amidoschwartz 10b dye. The successful separation of human parotid saliva by paper electrophoresis as described in this paper suggested a number of questions. Are there changes in parotid saliva electrophoretograms among individuals in both health and disease states at various times? What is the reason for the peculiarities in affinity for specific dyes in certain zones? What are the chemical natures of the components of the various bands? What are the potential antimicrobial activities of certain components? Present studies are seeking answers to these questions.

SUMMARY

Preliminary work indicated that application of electrophoresis designed for serum analysis is unsatisfactory when applied to the analysis of human parotid saliva. The main variation from other published techniques was the use of a 30-minute period which allowed the paper strips to attain an optimum amount of dampness. The most favorable results were obtained by conducting the electrophoresis at 21° C. and staining the strips with amidoschwartz 10b. By the use of this method it is possible to obtain clear-cut and distinct separations of eleven components in parotid saliva.

Grateful acknowledgment is made to Dr. Ned B. Williams for his assistance in this project.

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Inhibition of Experimental Caries by Sodium Metabisulfite and Its Effect on the Growth and Metabolism of Selected Bacteria

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In the course of a study which involved the sterilization of a cariogenic diet for use with germ-free animals, a study was first made of the effects of autoclaving this diet on the growth and caries experience of conventional (non-germ-free) white rats. Some of the results have been presented in a preliminary report.[1] Subsequently, as a result of modifications intended to inhibit the "browning reaction"[2] which results from the autoclaving of the milk-powder component of the diet, it was observed that the inclusion of 0.9 per cent sodium metabisulfite in the control unautoclaved diet resulted in markedly decreased caries scores. Furthermore, the compound seemed well tolerated by the rats. These observations prompted a further investigation into the use of this compound against experimental caries and also of its in vitro effects on selected microorganisms.

EXPERIMENTAL METHODS

Animal experiments.—Weanling female Sprague-Dawley rats from the NIH animal colony were used. They were 21 days old and weighed 35–50 gm. The animals were randomly distributed in groups of 3 in screen-bottom cages. The cariogenic diet employed was coarse corn-particle diet 585 described by Stephan *et al.*[3] Distilled water was available at all times.

In the initial experiment, sodium metabisulfite (Mallinckrodt) was ground to a fine powder with mortar and pestle. This was blended into the whole-milk-powder component before the complete diet was compounded, to give a final concentration of 0.9 per cent. Heat treatment consisted of 30-minute autoclaving at 120° C. in open shallow pans. Twelve rats were fed this diet, and 12 received unheated diet 585 containing the same concentration of sodium metabisulfite. Two additional groups of 12 rats each were fed the base diet 585, heated and unheated, as controls.

Body-weight gains were recorded weekly, and determinations of food intake were made at 1, 6, and 12 weeks.

At the end of the 90-day experimental period the animals were sacrificed. The heads were autoclaved to remove soft tissues, and the teeth were scored for occlusal caries, using essentially the system of Cox *et al.*[4] Examinations were conducted under a dissecting microscope with a sharp dental explorer.

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In a second experiment, decreasing concentrations of sodium metabisulfite were tested in order to correlate the metabisulfite level with caries scores. The powdered compound was added directly to the diet at 0.9, 0.3, and 0.15 per cent final concentrations. There were 24 animals per group. Other experimental details were the same.

Bacteriological experiments.—Concurrently with the caries studies, sodium metabisulfite was tested against the in vitro growth of a selected group of microorganisms, in order to determine inhibitory levels. Microbial strains representative of some of the oral types from humans and rats were employed.

Lactobacilli were grown in a medium of the following percentage composition: Casitone,* 1.2; yeast extract,* 0.5; sodium acetate, 0.2; Tween 80,† 0.1; KH_2PO_4, 0.5; salt solution, 0.2; glucose, 2.0. The salt solution contained $MgSO_4 \cdot 7H_2O$, 11.5 gm.; $MnSO_4 \cdot 4H_2O$, 2.5 gm.; $FeSO_4 \cdot 7H_2O$, 0.68 gm.; and distilled H_2O, 100 ml. The medium was adjusted to pH 6.2. Diphtheroids were also grown in this medium.

The composition of the medium used for the growth of streptococci was horse-meat infusion, 1,000 ml.; Trypticase (BBL), 10 gm.; NaCl, 5 gm.; glucose, 5 gm. The pH was adjusted to 7.1.

Trypticase-soy broth (BBL) was employed for the growth of the *Staphylococcus* and *Neisseria* strains.

Brain-heart infusion* with 0.2 per cent yeast extract was used for the growth of leptotrichiae.

Candida strains were grown in Sabouraud's dextrose broth at pH 5.7.

Five strains of *Veillonella* were tested. They were grown in a modified V-17 medium[5] without thioglycollate or basic fuchsin. Its per cent composition was Trypticase (BBL), 0.5; yeast extract, 0.3; NaCl, 0.5; Tween 80, 0.1; sodium lactate (60 per cent), 2.0; and the pH was 7.5.

All media except the V-17 medium were dispensed in 10-ml. amounts in 20 × 150-mm. cotton-stoppered tubes and autoclaved at 120° C. for 10 minutes. The V-17 medium was dispensed in 15 × 125-mm. screw-cap vials and sterilized in the same manner. Before use, V-17 medium was steamed in the Arnold sterilizer to drive off dissolved oxygen.

A solution of sodium metabisulfite was sterilized separately by filtration through a Corning UF sintered-glass filter. Concentrations of double-strength medium, sterile distilled water, and sterile sodium metabisulfite solution were adjusted to give 15 ml. of single-strength medium containing 0.4 per cent sodium metabisulfite in tube 1. Serial threefold dilutions were prepared by the aseptic transfer of 5 ml. through 7 tubes. A control tube was included.

Inocula for the tests were derived from 24-hour cultures in the same medium. Broth cultures were centrifuged, resuspended in sterile distilled water, and diluted 1:20, except for the veillonellae, which were not diluted. One drop per tube served as the inoculum. Incubation was at 37° C. for 18–20 hours. Growth was measured as optical density at 660 mμ on a Beckman B spectrophotometer.

Fermentation studies.—In addition to the determination of the inhibitory effects of sodium metabisulfite on the growth of selected organisms, studies were made of its

* Difco Laboratories Inc.

† The Atlas Powder Company.

effect on glucose metabolism at concentrations below those required for growth inhibition. For these studies *Streptococcus* strain Fa-1 was employed. This strain had been isolated from oral swabbings of rats on cariogenic diet 585 and has been provisionally classified as a strain of *Streptococcus mitis*. Cultures were grown in 250-ml. bottles with ground-glass stoppers in a medium of the following composition: Trypticase (BBL), 5 gm.; yeast extract, 5 gm.; K_2HPO_4, 5 gm.; salt solution, 0.5 ml.; distilled water, 500 ml. The salt solution contained $MgSO_4 \cdot 7H_2O$, 0.8 gm.; $FeCl3 \cdot 6H_2O$, 0.04 gm.; $MnCl_2$, 0.012 gm.; and distilled water, 100 ml. The pH of this double-strength basal medium was adjusted to 7.1. Each bottle contained 75 ml. of the basal medium and sufficient distilled water that addition of filter-sterilized solutions of glucose and sodium metabisulfite subsequent to autoclaving the basal medium made a final volume of 150 ml. The final concentration of glucose was 1 per cent.

TABLE 1

EFFECTS OF ADDING SODIUM METABISULFITE TO HEATED AND UNHEATED DIET 585

	Base Diet			
	585	585	585	585
Na metabisulfite	0	0	0.9%	0.9%
Autoclaved	−	+	−	+
Survivor ratio	10/12	9/12	12/12	6/12
Final body weight (av.)	199.9 gm.	53.0 gm.	191.2 gm.	41.0 gm.
Daily food intake (av.)	9.3 gm.	3.2 gm.	10.5 gm.	2.9 gm.
Carious teeth per rat	4.7	6.2	4.0	7.3
Carious surfaces per rat	8.3	13.2	4.3	12.3
Caries score per rat	19.1	30.9	5.2	25.0
S.E.	±2.04	±6.01	±0.92	±2.63

Inoculum was 2 drops from an 18-hour broth culture in the same medium, which had been centrifuged and resuspended to original volume in sterile distilled water. Incubation was at 37° C. for 18 hours.

Preliminary studies showed that the lower limit of complete inhibition under these conditions was approximately 0.01 per cent sodium metabisulfite. In the experiment to be reported, cultures were grown at 0.008, 0.006, and 0.005 per cent sodium metabisulfite. A control culture of unsupplemented medium was included. Measurements were made of growth and pH. Glucose utilization was calculated from residual glucose as measured by glucose oxidase,[6] using the Glucostat reagents and colorimetric procedure.* Lactic acid was measured by the method of Barker and Summerson.[7]

RESULTS

Animal studies.—The effects of sodium metabisulfite in autoclaved and unautoclaved diet 585 are compared in Table 1. This compound at a concentration of 0.9 per cent in the unautoclaved diet caused a marked reduction in the severity of caries normally expected on this diet. Values for body-weight gains, food intake, and survivor

* Worthington Biochemical Corporation, Freehold, N.J.

ratios were essentially comparable to the controls. Addition of sodium metabisulfite to the diet before autoclaving did not appear to protect it from heat damage; in fact, the animals fared better on the heated control diet without sodium metabisulfite. The mortality in the former group was 50 per cent, as compared with a mortality of 25 per cent for the latter group. From a study of glucose–amino acid model systems, Friedman and Kline[8] have concluded that the use of browning inhibitors does not give complete assurance of retention of nutritive quality. Increased caries was observed in the groups on autoclaved diets. This has been reported by others.[9, 10]

The effects of varying the concentration of sodium metabisulfite were next studied. The results presented in Table 2 confirm the reduction of caries on the unautoclaved diet containing 0.9 per cent sodium metabisulfite. The inclusion of 0.3 and 0.15 per cent sodium metabisulfite in the diet resulted in correspondingly less caries reduction. These three concentrations gave reductions of 77, 64, and 33 per cent, respectively. In

TABLE 2

INHIBITION OF OCCLUSAL FISSURE CARIES WITH DECREASING CONCENTRATIONS OF SODIUM METABISULFITE

	Base Diet (Unautoclaved)			
	585	585	585	585
Na metabisulfite	0	0.9%	0.3%	0.15%
Survivor ratio	24/24	23/24	21/24	23/24
Final body weight (av.)	203.7 gm.	190.6 gm.	195.4 gm.	205.3 gm.
Daily food intake (av.)	9.0 gm.	10.9 gm.	10.6 gm.	10.9 gm.
Carious teeth per rat	3.8	2.2	2.5	3.3
Carious surfaces per rat	7.5	2.6	3.1	5.1
Caries score per rat	13.8	3.1	4.8	9.3
S.E.	±1.95	±0.63	±1.33	±1.80

this experiment as in the previous one, there was a slight increase in food consumption when sodium metabisulfite was added to the diet and also a slight reduction in body-weight gains at the higher concentrations.

In vitro antimicrobial activity.—The effect of sodium metabisulfite on the growth of *Streptococcus salivarius-3 in vitro* is illustrated in Figure 1. This strain was completely inhibited at 0.4 per cent, but increasing growth occurred at 0.133 per cent and at concentrations below this. The end point for complete inhibition was estimated by extrapolation to be 0.3 per cent. Inhibitory end points for all other organisms tested were obtained in this manner and are listed in Table 3, along with end points for 50 per cent inhibition. A linear response to the inhibiting agent was also observed in the case of *S. salivarius* 9756, *Lactobacillus fermenti* 36 and NR 77, and diphtheroid strains D1213 and D1025. For all other organisms the curve was non-linear, suggesting the interplay of a number of factors which may vary the inhibitory response at different metabisulfite concentrations. In some cases, as shown in Table 3, there was actually stimulation of growth at the lower concentrations of sodium metabisulfite. This may be partly due to its reducing properties.

The lactobacilli were generally more sensitive to sodium metabisulfite than were

most of the streptococci. However, the two rat *Streptococcus* strains tested were exceptions, being about as sensitive as the lactobacilli to this agent.

Inhibition of the *Candida* strains seemed to vary with the species tested. *Candida albicans,* the pathogenic species, was less sensitive than *C. krusei.* The latter species was markedly stimulated by lower concentrations of sodium metabisulfite. The sensitivity of *C. pseudotropicalis* was intermediate between the other two species.

The leptotrichiae and the diphtheroids seemed to be the most sensitive of the organisms tested.

Effects on carbohydrate fermentation.—For studies of the effects of sodium metabisulfite on the metabolism of carbohydrates, the rat oral *Streptococcus* strain Fa-1 was selected. Even below growth-inhibitory concentrations, sodium metabisulfite apparently exerted a marked effect on the glucose metabolism of this *Streptococcus.* The results

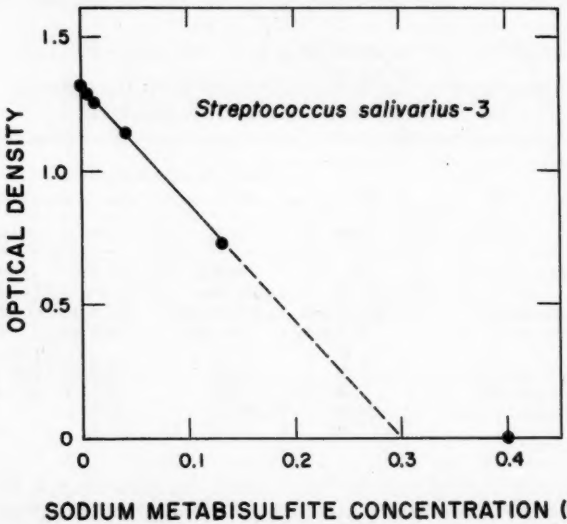

FIG. 1.—Inhibition of growth of *Streptococcus salivarius-3* by sodium metabisulfite. The broken line represents extrapolation to the 100 per cent inhibition concentration.

are summarized in Table 4. There was a decrease in total acidity measured as pH. Lactic acid production was also decreased. The ratio of lactate formed to the amount of glucose utilized in the metabisulfite series indicates a shift from the normal homofermentative pattern of this organism. At 0.005 per cent metabisulfite—the lowest concentration tested—there was a slight stimulation in growth as evidenced by an increased optical density of the culture and an increase in the amount of glucose utilized. Even under these conditions there was quantitatively less acid produced than there was by the control. This growth stimulation was observed in repeated experiments. Further studies on the distribution of fermentation end products by this organism under the influence of sodium metabisulfite are now in progress.

DISCUSSION

Although it is difficult to estimate the effective concentration of any agent in the microbial plaque or even in the oral cavity as a function of its concentration in the

TABLE 3

INHIBITION AND STIMULATION OF SELECTED MICROORGANISMS BY SODIUM METABISULFITE

Microorganisms	Strain No.	Stimulatory Concentration	50% Inhibition Concentration	100% Inhibition Concentration*
Lactobacillus arabinosus	17-5	.....	0.025	0.0325
L. plantarum	8014	.....	.0275	.040
L. casei	7469	.....	.021	.030
L. fermenti	36	.....	.010	.020
L. fermenti (rat)	NR77	.....	.025	.050
L. acidophilus (rat)	42T	.....	.045	.068
Candida albicans	358	.....	.102	.40
C. albicans	394	.....	.087	.40
C. pseudotropicalis	360	.....	.080	.150
C. krusei	359	0.0050	.055	.133
C. krusei	379	.0015	.030	.054
Neisseria catarrhalis	N6	.....	.036	.060
N. flava	N8	.0050	.034	.053
Diphtheroid	D1213	.....	.0075	.015
Diphtheroid	D1025	.....	.00025	.005
Diphtheroid	D1201	.006	.023	.028
Diphtheroid	D688	.006	.013	.015
Streptococcus salivarius	9756	.....	.20	.40
S. salivarius	3	.....	.15	.30
S. faecalis	R	.....	.175	.40
S. mitis	2	.....	.125	.25
Streptococcus (rat)	Fa-1	.0046	.0112	.018
Streptococcus (rat)	3R-114	.0046	.022	.036
Veillonella (rat)	23	.....	.035	.050
Veillonella (rat)	25	.....	.024	.0325
Veillonella (rat)	22X	.....	.019	.030
Veillonella	CAM	.....	.024	.0325
Veillonella	SEW	.....	.024	.0325
Staphylococcus aureus	13	.....	.09	.190
S. aureus	33	.0050	.03	.055
Leptotrichia	L891	.0005	.016	.018
Leptotrichia	L1187	.0005	.016	.018
Leptotrichia	L890	.0005	.006	.010
Micrococcus	19	0.0050	0.120	0.17

* Estimated graphically by extrapolation, per cent sodium metabisulfite in the medium.

TABLE 4

EFFECTS OF SODIUM METABISULFITE ON GLUCOSE FERMENTATION BY RAT STREPTOCOCCUS

Per Cent Metabisulfite	Growth as O.D. at 660 mμ	pH	Mg Glucose Used*	Mg Lactic Acid Produced*	Molar Ratio Lactate/Glucose
0	1.59	4.4	780	617	1.58
0.008	1.11	6.2	525	264.5	1.01
0.006	1.59	5.0	720	362	1.01
0.005	1.64	4.6	840	437	1.04

* 150-ml. culture incubated at 37° C. for 18 hours. Readings corrected for blank values of uninoculated medium in each case.

diet, caries reduction in rats by sodium metabisulfite may be due at least in part to the inhibitory potential of this compound on the oral flora. The concentration range in the diet, 0.9–0.15 per cent, which inhibited caries in vivo, was not much higher than the concentration required to inhibit the growth of most microorganisms in vitro. While tests of this type for the in vitro inhibition of bacteria may vary, depending on the type of medium employed, size of inoculum, incubation time, and other factors, they do give a sufficiently general idea of the relative sensitivities of the different bacteria for the purposes of this study.

Sodium bisulfite and also sodium metabisulfite were shown by Dreizen *et al.*[11] to reduce acid production in saliva both in vitro and also by the use of troches in vivo; however, these authors did not attempt to distinguish between the antibacterial and antienzymatic properties of this compound. On the basis of the data presented in the present work, it would appear that sodium metabisulfite exerts a dual action on the oral microflora. At higher concentrations there is a marked inhibition of growth, and at subinhibitory concentrations, where growth is not affected, acid production may be inhibited. A sulfite effect on yeast fermentation was observed by Neuberg,[12] in which the normal alcohol fermentation is diverted to a glycerol fermentation. Sulfite formed an addition compound with acetaldehyde, preventing the reduction of this intermediate to ethanol. The regeneration of DPN from reduced DPN normally accompanying this reaction proceeded by an alternate pathway, resulting in the production of glycerol. Bisulfite exerts a similar effect on glycolysis in muscle,[13] although different intermediates are involved. It would seem from the present work that metabisulfite may influence glycolysis in lactic acid bacteria, causing a shift away from homofermentation.

SUMMARY

Incorporation of sodium metabisulfite into a coarse corn-particle, high-sugar diet resulted in a marked reduction in occlusal fissure caries in white rats. Addition of 0.9, 0.3, and 0.15 per cent sodium metabisulfite reduced caries scores by 77, 64, and 33 per cent, respectively. This compound was also inhibitory to the growth in vitro of a variety of microorganisms, including lactobacilli, streptococci, staphylococci, micrococci, candidae, leptotrichiae, diphtheroids, neisseriae, and veillonellae. Even in subinhibitory concentrations of sodium metabisulfite there was an apparent interference with the glucose metabolism of a streptococcus isolated from the rat. This was manifested in a decreased acid production under conditions of equivalent or slighlty stimulated growth.

The technical assistance of William L. Poole and Henry Burris in the animal experiments is gratefully acknowledged. *Leptotrichia,* diphtheroid, and *Candida* strains were obtained from Dr. Arden Howell, Jr. Strains of *Streptococcus salivarius* and *S. mitis* were obtained from Dr. Thomas A. Nevin.

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The Tubercle of Carabelli in the Kish Dentition, Mesopotamia, 3000 B.C.

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The tubercle of Carabelli, also known as "tuburculum anomalum" or "tuberculum impar," was described by an Austrian dentist, Georg Carabelli, in 1842. The tubercle is situated on the lingual surface opposite the anterointernal cusp of the upper permanent molars. Sicher[1] described the tubercle as an "accesory cusp." The tubercle appears in varying sizes from a well-developed cusp to a depressed grooving on the same location. The cusp is generally found on the upper first molars and very rarely on the third molars. The tubercle, when found on the second molar, does not present the same degree of prominence as its mesial counterpart. Its presence has been generally observed as bilateral and rarely unilateral. Bolk[2] observed that the tubercle frequently occurred on the deciduous second molars.

Considerable investigation has been done on the incidence, morphology, and evolutionary significance of Carabelli's cusp. Reports written by previous investigators have been valuable in the analysis of the incidence of the cusp in the Kish dentition (Table 1). The purpose of this paper is to present information concerning the role of the tubercle of Carabelli in the Kish molars from the evolutionary point of view and as a racial characteristic in tooth morphology.

Adloff[3, 4] observed the cusp in Krapina molars. He concluded that it is a primitive human characteristic because of its rarity in the anthropoids. Many authors recognized the cusp as a remnant of the basal cingulum. Weidenreich[5] discounted Adloff's observation, stating that there were no cusps on the Krapina molars, but pits representing cusps. Weidenreich[5] observed the absence of the tubercle in the *Sinanthropus* molars. Gorjanovic-Kramberger[6, 7] also reported the presence of the cusp in Krapina molars. Robinson[8] observed cusps and pits on the first, second, and third molars of the Australopithecines.

Dietz[9] made a survey of the incidence of the various types of cusps in 732 American Army men unidentified as to racial descent and found the cusp to occur in 72.3 per cent of the men. Bolk[2] reported 17.4 per cent completely developed cusps and 44.2 per cent incompletely developed cusps in the 2,325 European children he studied. Batujeff[10] found that 50 per cent of the Russian children he examined exhibited the cusp on their maxillary molars. De Terra[11] found 6.45 per cent fully developed cusps and 11.6 per cent incompletely developed cusps in recent Europeans. Diamond[12] mentioned in his anatomy textbook that the tubercle appeared on 50 per cent of the upper molars and that it was bilateral when present. Dahlberg[13] found that 41 per cent of the

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TABLE 1

INCIDENCE OF TUBERCLE OF CARABELLI IN DIFFERENT POPULATIONS

POPULATIONS	INCIDENCE (PER CENT)		
	M^1	M^2	M^3
Dutch children (Bolk)	17.4	21.7	.....
Russian children (Batujeff)	50	.....	.....
Recent American whites (Dahlberg)	41	8	0
American army men (Dietz)	72.3	.....	.....
Yugoslavian children (Kallay)	52.13	.....	.....
American children of northwestern European descent (Meredith)	84	.....	.....
Mixed Europeans (Diamond)	50	.....	.....
Kish (present findings)*	24	2.4	0
Recent Europeans (De Terra)	6.45 fully developed cusp; 11.6 incompletely developed cusp		
European children (Bolk)	17.4 fully developed cusp; 44.2 incompletely developed cusp		
Bantu (Shaw) skulls (5-141)†			
Bantu (Shaw) living (4-248)†			
East Greenland Eskimo (Pederson)	0	0	0
Pima Indians (Dahlberg) Cusps	8	0	0
Pima Indians (Dahlberg) Pits	27	4	0
Modern Chinese (Oshima)	2.1	.....	.....
Japanese (Hirakawa)	10.7	.....	.....
Aleut (Moorrees) (8–60)†	.....	.....	.....

* The Kish figure represents only the right molars.

† The numbers inclosed in parentheses represent the actual figures reported by the investigators, as: (5-141), 5 out of 151 skulls.

FIG. 1.—Maxillary first molar of Kish #192677, showing Carabelli's cusp

recent American whites exhibit the cusp on their first maxillary molars and 8 per cent on the second molars. Meredith and Hixon[14] made a survey of the incidence of the tubercle in 200 American children of northwestern European origin; 84 per cent of the molars they examined showed the cusp in varying sizes, and 16 per cent had no cusp. Kallay[15] also found the cusp in 52.13 per cent of Yugoslavian children.

Shaw[16] observed that the cusp occurred bilaterally in the upper molars in five of the Bantu skulls he examined. He also observed the presence of the cusp in 4 of 248 living Bantus. Shapiro[17] reported that the tubercle is more frequently found in the Negroid than in the Mongoloid race. Pederson[21] did not find any trace of the cusp in the East Greenland Eskimo skulls. Dahlberg[13] reported an 8 per cent incidence of the cusp on the second and third molars. Pits were observed on 27 per cent of the maxillary first molars, 4 per cent on the second molars, and none on the third molars. Moorrees[18] observed only 8 out of 60 Aleuts to have the cusp on their molars. Oshima[19] reported a

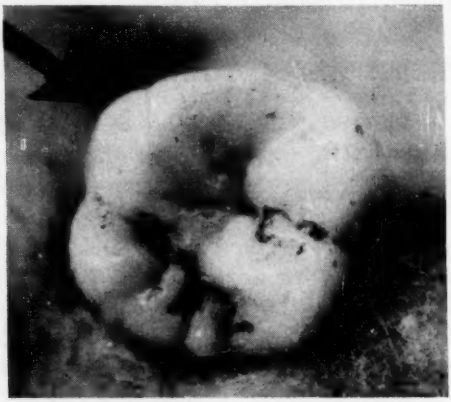

FIG. 2.—Slight suggestion of Carabelli's complex on Kish #192636

low incidence of the cusp in the Mongol race; he found only 2.1 per cent incidence of the cusp in 1,250 modern Chinese from Manchuria. Hirakawa[20] stated that he found a 10.7 per cent incidence of the cusp in 946 Japanese.

PRESENT MATERIALS AND FINDINGS

The data gathered for this paper were collected from skulls from Kish, Mesopotamia, 3000 B.C. This report on the tubercle of Carabelli in the Kish dentition is part of a paper written on the morphology of the dentition of the people from Kish, 3000 B.C. This work was carried out at the Chicago Natural History Museum, where the collection is kept.

The number of teeth available for morphological scrutiny was small. The major part of the collection is not well preserved and has deteriorated considerably. For this report, the right and left molars were counted separately.

Forty-seven right and 48 left maxillary molars were examined. Twenty-three per cent of the right molars exhibited the cusp in varying sizes, and in 24.4 per cent of the left molars the cusp was observed in the same variation as the right molars. In only one case was a fully developed cusp observed bilaterally on the first and second molars.

The result gathered from the second molars was 2.4 per cent, for the right and left sides; 45 right, and 36 left molars were examined for this purpose.

No tubercle of Carabelli was observed on any of the 28 right and 24 left third molars.

DISCUSSION

Previous literature written on the tubercle of Carabelli, concerning evolutionary and population studies, is important in the discussion of the present findings.

Comparison of the results gathered from the modern populations of European origin and those gathered from Kish and other human representatives earlier than Kish suggests a definite state of development of the tubercle in the morphological structure of the human dentition, 3000 B.C., in the Kish people. The appearance of the cusp and pit in the Australopithecines as reported by Robinson,[8] and the occurrence of the same in the Neanderthals (Krapina) observed by Garjanovic-Kramberger[6,7] are evidences of the evolutionary development of the tubercle in the maxillary molars. The Carabelli complex is unknown in pongids and rare in fossils. Robinson[8] believes that it is a common feature in the euhominids from the Australopithecines to the present and therefore has an evolutionary significance.

From the racial point of view, the tubercle of Carabelli is a trait that has a low incidence in the Mongoloid race. Weidenreich's[5] statement of its absence in the *Sinanthropus* molars strongly contends that it is a trait absent in the Mongoloid race, its occurrence in the modern Mongoloid races being the result of genetic factors such as racial interbreeding and migration. The low incidence of the cusp in the Mongoloid race and its subraces is substantiated by the results gathered from the Pima Indians, the Aleuts, the modern Chinese, and the Japanese. Pederson[21] found no Carabelli trait in the East Greenland Eskimo skulls. Korkhaus[22] pointed out that the Carabelli trait is inherited as a dominant Mendelian character. Kraus[23] also worked on the genetics of this trait. More evidence is needed to support this statement; however, Dietz[9] stated that its inheritance may occur in various degrees of expression.

SUMMARY

1. The incidence of the tubercle of Carabelli in the Kish dentition, 3000 B.C., indicates an evolutionary development of the cusp in the morphology of human dentition.

2. The comparative data gathered from the American, European, and Mongoloid people reveals the fact that the Carabelli cusp is a trait inherent largely in the modern white people of European origin.

3. The absence or low incidence of the cusp in the Mongoloid groups indicates that genetic mechanisms are involved in its occurrence in the mentioned populations.

The author wishes to acknowledge and extend her thanks to the Chicago Natural History Museum, to Dr. Paul Martin and to his staff for their courtesies and the use of their facilities in studying the Museum's excellent skeletal collection of the Kish population. Also, appreciation is extended to Dr. Albert A. Dahlberg of the Zoller Memorial Dental Clinic.

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The Salivary Glands in Lysine-deficient Young Rats

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Previous studies in this laboratory have shown that a lysine supplement will inhibit dental caries induced in rats by a purified zein-base diet,[1] as well as by diets which were lysine-deficient because of their content of heat-processed cereal foods[2] or skim-milk powders.[3-5] The purpose of these studies was to determine whether pathologic changes ocurred particularly in the salivary glands of young rats on lysine-deficient and lysine-limited diets. Salivary function was evaluated also in terms of total nitrogen, protein, tyrosine, and "tryptophan"* in salivary specimens obtained prior to sacrifice. In a repletion study, rats were deprived of lysine for 30 days and then given a diet containing 1 per cent lysine for 30 days. This approach was suggested by the observation in man that enlargement of the salivary glands may occur during recovery from a period of malnutrition.[6] This study provided an opportunity to investigate other systemic effects and to correlate the data with observations associated with lysine deficiency in the rat, as previously reported[7-9] following the classical original research in this field by Osborne and Mendel.[10]

MATERIALS AND METHODS

The general plan of three series of studies comprosing this report is apparent in Figures 1, 2, and 3, which present the average growth data of the experimental rats. The composition of the diets has been published in detail elsewhere.[1, 3]

In Study I four littermate rats at weanling age were taken from each of ten litters and divided into four groups (*A, B, C, D,* Fig. 1), equally divided by number and sex. Group A was on experiment 60 days; Groups B, C, and D, 30 days. Groups A and B received the zein-base lysine-deficient diet LA. On the basis of previous experience, some mortality was anticipated in Group A. Thus four animals were added to this group. Groups C and D received the lysine-adequate diet LB. The ten pairs of rats comprising groups B and C were pair-fed—a regimen in which the rat consuming the least quantity of diet (in this case diet LA) established the level of food intake for the pair. Group A (diet LA) and Group D (diet LB) ate ad libitum.

In Study II (Fig. 2) the effect of lysine repletion after 30 days on lysine-deficient diet LA was observed in Group E rats by comparison with control Groups F and G, each containing ten rats fed the adequate lysine diet LB. Paired rats in Groups E and

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* Ehrlich-positive substances.

F received equal quantities of food, whereas Group G rats ate ad libitum. In addition, the Group A rats (Fig. 1) which received ad libitum diet LA for a period of 60 days were used for comparative pathologic study, although they were not littermates of rats in E, F, and G. Each group contained the same number of males and females. Several extra animals were added to assure at least ten in each group at the termination of the experiment.

In Study III (Fig. 3), twenty female littermates in each group (H and I) received diet ad libitum for 60 days, starting at weanling age. As indicated by the rate of growth, diet 6.36—an autoclaved skim-milk-powder diet—was growth-limiting in lysine,*

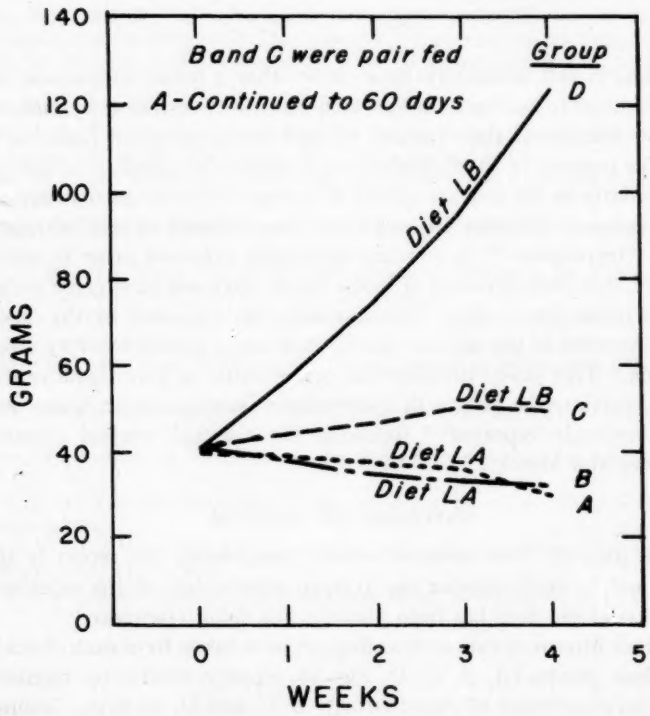

FIG. 1.—Growth records of rats lysine-deficient for 30 days, Groups A and B; normal controls, C, pair-fed with Group B; controls, Group D, fed ad libitum.

whereas diet 636-1 contained a 2.0 per cent L-lysine supplement. This study was initiated 3 months after termination of Study II when sufficient pathologic reports from Studies I and II indicated no sex differences in the salivary gland findings. The weight records of the Group A rats are included in Figure 3 for comparative purposes.

All the rats were of the Sprague-Dawley strain from the National Institutes of Health colony. They were housed in individual cages and drank distilled water ad libitum. Just prior to sacrifice, pilocarpine-stimulated saliva was collected[11, 12] from rats in Groups A, B, C, D, H, and I, and 1.0-ml. aliquots of the pooled specimens from each group were analyzed for total nitrogen by Kjeldahl, and 1 ml. of a 1:50 dilution was

* Microbiologically this diet assayed 11.2 mg. lysine per gram of diet.

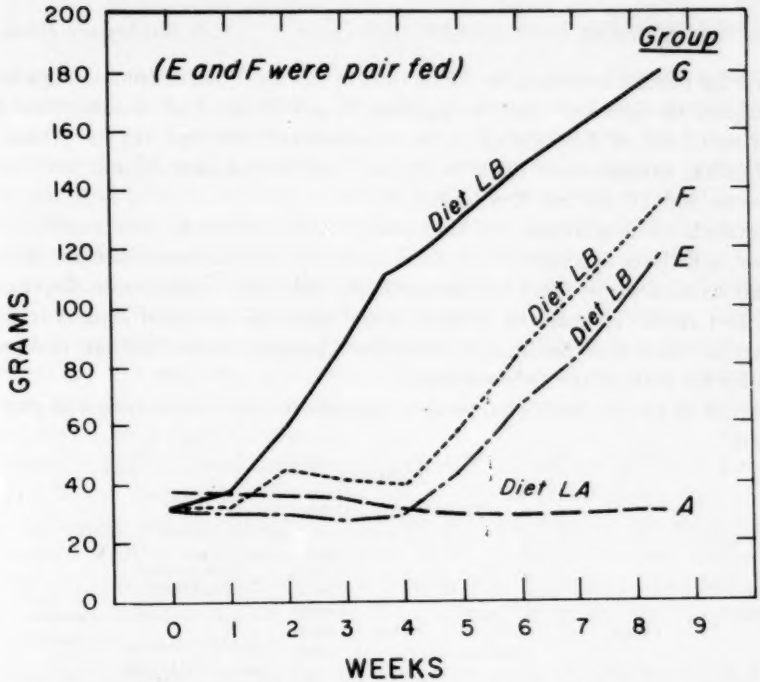

FIG. 2.—Growth records of rats repleted after 30 days of lysine deficiency, Group E; normal controls, F, pair-fed with Group E; ad libitum fed controls, Group G; 60-day lysine-deficient, Group A.

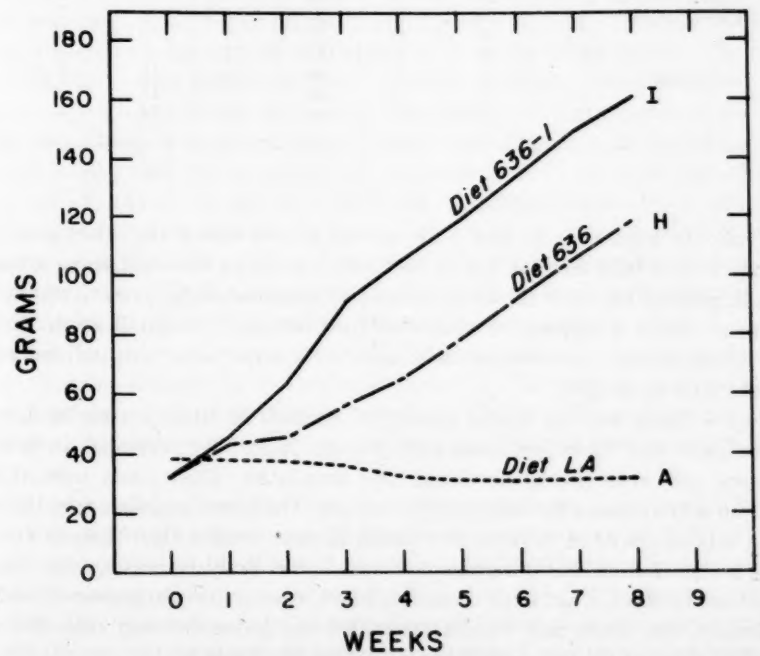

FIG. 3.—Growth records of rats on lysine-limited diet of milk powder, Group H; controls with lysine supplement, Group I, and 60-day lysine-deficient, Group A.

analyzed for protein according to the method of Lowry.[13] Saliva from Groups H and I was analyzed for tyrosine[14] and "tryptophan"[15] as follows: 2 ml. of saliva were hydrolyzed with 1.0 ml. of 2 N NaOH in an autoclave at 2 atmospheres for 1 hour. After neutralization, samples were made to 5.0 ml., centrifuged, and 2.0 ml. were analyzed for tyrosine and 1.0 ml. for "tryptophan."

All animals were sacrificed by chloroform. Gross pathology was recorded during necropsy, and tissue specimens were fixed in 10 per cent buffered formalin. Histologic preparations were made, using routine methods, and slides stained with Mayer's hematoxylin and eosin. Tongue and salivary gland sections were also stained with PAS-hematoxylin, some with preliminary disastase digestion. Frozen sections of liver were stained for fat with oil-red O-hematoxylin.

The teeth of all rats were examined for smooth-surface dental caries, as previously described.[2]

TABLE 1

PATHOLOGIC FINDINGS

Group		Total No. of Animals	Deaths	Duration of Experiment (Days)	"Spontaneous" Pathology		Acute or Chronic Glossitis
					No. of Animals Affected	No. of Organs Affected	
Lysine deficient	A	14	4	60	5	6	6
	B	10	0	30	3	3	2
Lysine repleted, E		10	0	60	3	5	2
Lysine limited, H		20	0	60	13	18	0
Lysine adequate	C	10	0	30	3	3	0
	D	10	0	30	2	3	1
	F	10	0	60	6	10	1
	G	10	0	60	8	13	3
	H	20	1	60	12	13	4

RESULTS

General.—In Figures 1, 2, and 3 the growth curves reflect the effect of a lysine-deficiency in zein-base diet LA and in diet 636. The latter diet containing autoclaved skim-milk powder has been shown previously to promote better growth with a lysine supplement. Under a regimen of controlled food intakes (Groups B versus C and E versus F) the lysine-supplemented diets apparently were better utilized and resulted in a better gain in weight.

Study I.—There were no deaths among the animals in Study I after 30 days on a lysine-deficient diet. However, these rats (Groups A and B) presented an unhealthy appearance and were poorly developed and emaciated. Their coats were thin and ruffled, and subcutaneous fat was scanty or absent. The bones and viscera of the lysine-deficient rats sacrificed at 30 days, i.e., Group B, were smaller than those of Group D, controls fed ad libitum, but otherwise not remarkable. Body fat was scanty. The pair-fed controls (Group C), although undernourished, were active and appeared fairly normal. Grossly, the bones and viscera resembled the lysine-deficient rats. No deaths occurred in either of the two control groups, C and D (Table 1).

Salivary glands, parotid.—Lysine-deficient animals in Group B revealed smaller acini and lining epithelium than in controls, Group D, on comparative microscopic examination (Figs. 4 and 5). The nuclear size and appearance were about the same, but the amount of cytoplasm and its constituents—i.e., chromotoid substance, reddish-violet granules, and PAS-positive material—were reduced. The latter was resistant to diastase digestion. The cytoplasm differed also in that it frequently contained coarse vacuoles in the lysine-deficient animals. Similar quantitative differences were observed in the ducts and their lining cells, i.e., smaller size and less cytoplasm in the ductal epithelium. Also, the nuclei differed, in that small, round, or oval hyperchromatic forms were observed only in the lysine-deficient. The gland stroma contained less fat in the lysine-deficient group. The majority of the pair-fed controls, Group C, were similar to the lysine-deficient group. There were no striking differences in the above histologic features between males and females.

Sublingual.—The acini and their lining epithelium, as well as ducts and ductal epithelium, were also decreased in size in the lysine-deficient group compared with Group D. The cytoplasm was less abundant, and PAS-positive droplets less numerous. The latter resisted diastase digestion. Small oval or round hyperchromatic ductal nuclei were observed only in the lysine-deficient animals. The majority of pair-fed controls, Group C, were similar to the ad libitum fed controls, Group D. No differences were observed in males and females.

Submaxillary.—Smaller acinar size and indistinct acinar outline were found in the lysine-deficient compared with control Group D. The cytoplasm of the mucoid acinar epithelium was less abundant, often coarsely vacuolated, and contained few PAS-positive droplets. The latter resisted disastase. In the ductal epithelium the cytoplasm was also less abundant. Small hyperchromatic nuclei were sometimes observed. Fewer PAS-positive droplets were apparent in the cytoplasm of the ductal epithelium. The pair-fed controls, Group C, were similar to Group D except in ductal development.

Tongue.—Much less serous and mucous glandular tissue was apparent in the lysine-deficient rats, Group B, compared with control Group D. The acini and acinar epithelium were smaller, and the cytoplasm was particularly reduced in amount within the mucous cells. In the serous cells it was coarsely vacuolated, basophilic, or neutrophilic and contained few pink granules by comparison with the large number of reddish-violet granules in the control. There were fewer PAS-positive granules within the cytoplasm of the serous cells, but bright-red droplets could be found in the mucous cells of the lysine-deficient. The PAS-positive material was resistant to diastase digestion. Two out of ten lysine-deficient animals showed focal subacute or chronic glossitis by contrast with one animal only in Group D (Table 1). There was no glossitis in Group C. The glandular tissue of the tongue in animals of Group C was intermediate in its development. No differences in the above histologic features were observed between sexes.

Bones and viscera.—The left femur and sternum were decalcified and prepared in routine fashion for microscopic study. Microscopic examination of hematoxylin and eosin stained sections of the femur revealed impaired development in the lysine-deficient on comparison with control Group D (Figs. 6 and 7). The trabeculae within the head of the femur were fewer and smaller. The epiphyseal plate at the head end of the bone was thinner, the cartilage cells irregularly arranged, and the plate covered with a

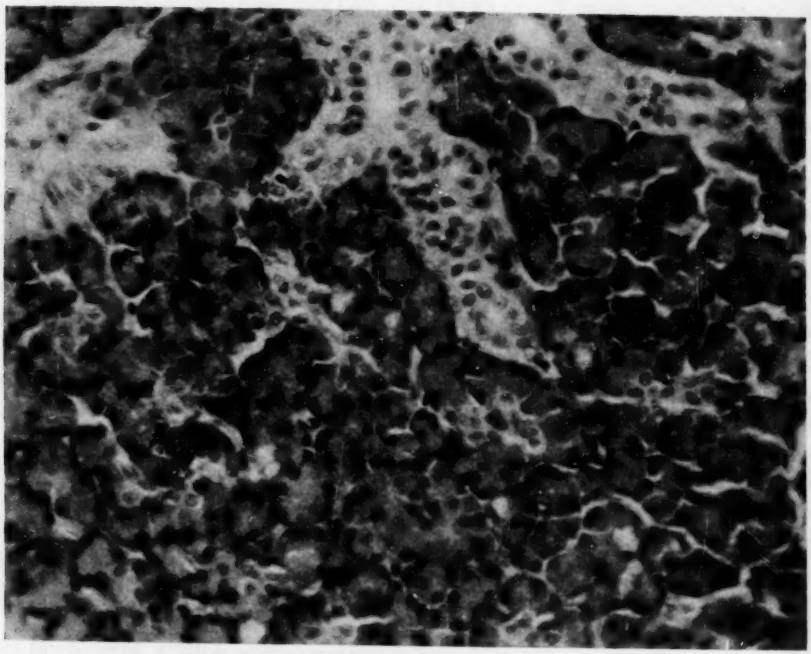

FIG. 4.—Normal parotid gland, rat fed ad libitum, Group D. ×285

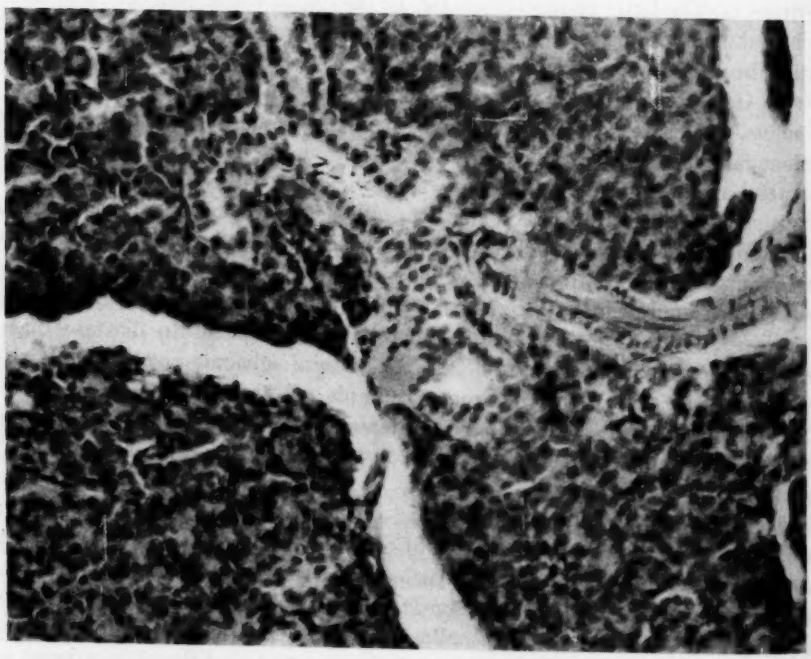

FIG. 5.—Parotid gland of rat, lysine-deficient 30 days, Group B. ×285

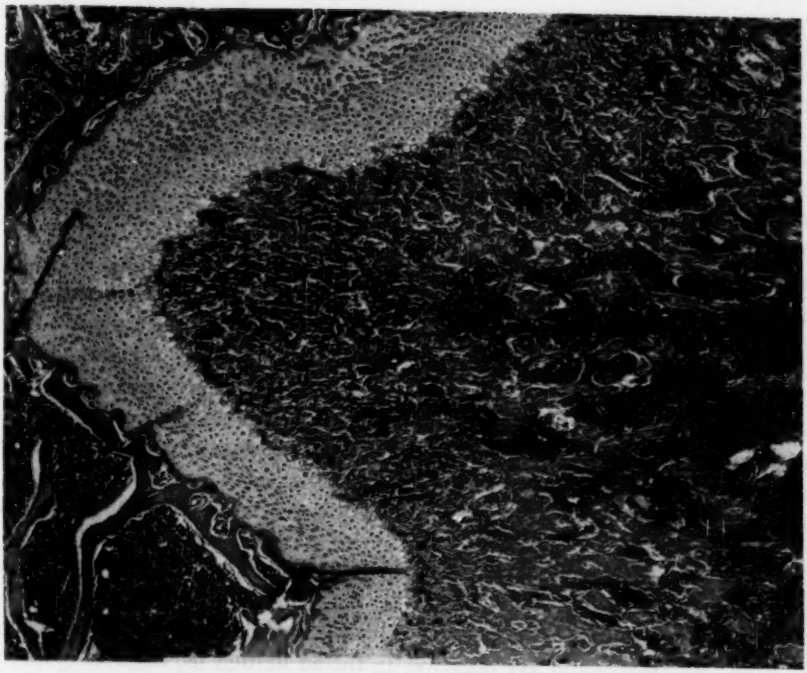

FIG. 6.—Normal epiphyseal plate, head of left femur, Group D. ×40

FIG. 7.—Same area left femur, lysine-deficient rat, Group B. ×40

thinner layer of bone on the head side. Only scattered delicate bony trabeculae were visible on the diaphyseal side, by contrast with many closely packed larger trabeculae in the control Group D. The cortex was also thinner, and the trabeculae of the medulla also less developed. The marrow revealed less erythropoiesis, and the myeloid-erythroid ratio appeared to be increased in the lysine-deficient. The pair-fed controls in Group C were intermediate between Groups B and D in their development. No differences in sexes were observed.

Bone development, cortical and trabecular, was better in the sternum of the normal control Group D than in the lysine-deficient rats. The marrow also revealed less erythropoiesis in the latter, and the myeloid/erythroid ratio was increased. The pair-fed control Group C was intermediate, and no differences in sexes were noted.

The spleen was usually smaller in the lysine-deficient, Group B, than in the ad libitum fed normal controls of Group D. Microscopically, the Malpighian corpuscles were smaller. In the stroma of the red pulp, hematopoiesis was less active, and erythroid and megakaryocytic elements decreased. The spleen in the pair-fed controls in Group C was small also and often showed changes similar to those described in the lysine-deficient.

The liver was smaller in the lysine-deficient than in the ad libitum fed normal controls of Group D. Although vacuolated cytoplasm seemed to be more frequent in the hepatic cords of the lysine-deficient, special fat stains revealed no differences: two were considered to display weak positive fatty metamorphosis in the lysine-deficient; three were questionable and five negative. In the ad libitum fed controls of Group D, three were negative, four questionable, and three weakly positive in this respect. The pair-fed controls were all negative or questionable.

Sexual development and maturation were impaired in the lysine-deficient rats. The pair-fed controls of Group C were intermediate in this respect. In all females in the lysine-deficient group the uterus, oviducts, and ovaries were difficult or impossible to identify grossly. Uterus and oviducts usually appeared as fine threadlike structures. The ovaries could not be identified with certainty. The testes, epididymides, and accessory glands were also smaller and immature compared to the ad libitum fed controls of Group D. In the latter the ovaries on microscopic examination were observed to contain developing, sometimes ruptured, follicles. In males, spermatozoa were numerous in the seminiferous tubules and epididymides of the ad libitum fed controls. In the lysine-deficient Group B no ovarian tissue could be found for microscopic study. Occasional sections of immature uterus were obtained. In the males on the lysine-deficient diet only rare spermatozoa were observed in the seminiferous tubules or epididymides.

"Spontaneous" pathology.—Three lysine-deficient animals in Group B revealed focal nonspecific acute inflammation—colitis, lymphadenitis, and pneumonitis, respectively (Table 1). In the ad libitum fed normal controls of Group D two animals revealed similar, non-specific inflammatory changes affecting a total of three organs: one with a lymph node abscess and pneumonitis and the other with pneumonitis alone. Three pair-fed controls of Group C showed non-specific interstitial nephritis, pneumonitis, and esophagitis, respectively. In the controls the inflammatory changes were focal and could not be correlated with systemic effects, insofar as general appearance or body weight was concerned.

Saliva.—The results of the salivary analyses are shown in Table 2. No marked dif-

ferences were observed in any of the rat groups for the constituents examined. Thus, irrespective of treatment, the total nitrogen varied from 0.13 to 0.25 per cent and the protein from 0.64 to 1.00 per cent.

Dental caries.—Very little dental caries was present in the rats comprising Study I, contrary to a previous experiment in which diet LA proved cariogenic at the end of 60 days.[1] In Study I, however, the number of rats was small (fourteen), and they were on the experiment only 30 days. In addition, these rats were housed individually, whereas dual housing has usually been conducive to caries production.[2] As was expected,[5] diet LB containing a lysine supplement was not cariogenic.

Study II.—In Figure 2 and Table 1 the data are indicated concerning growth, mortality, and pathologic features in Study II. The parotid, sublingual, and submaxillary glands and tongue were examined microscopically as in Study I. The 60-day lysine-deficient animals, Group A, revealed about the same degree of impaired development in all salivary gland tissue as was observed in the hematoxylin and eosin and

TABLE 2

PROTEIN, NITROGEN, TYROSINE AND "TRYPTOPHAN," ANALYSIS OF POOLED RAT SALIVA

	Group No.					
	A	B	C	D	H	I
Diet	LA	LA	LB	LB	636	636-1
Days on experiment	60	30	30	30	60	60
Lysine supplement	−	−	+	+	−	+
Total N (%)	0.16	0.16	0.25	0.13	0.14	0.15
Protein	0.67	0.64	1.00	0.65	0.80	0.80
Tyrosine (mg/%)					15.4	15.4
Tryptophan (mg/%)					18.8	19.8

PAS-stained sections of the 30-day lysine-deficient Group B. No evidence of progression to the point of atrophy, necrosis, or fibrosis could be demonstrated. However, glossitis was somewhat more frequent in the 60-day lysine-deficient.

Group E, lysine-deficient for 30 days and then repleted for the next 30 days, showed restoration to a comparable degree with both control Groups F and G. There was no evidence of reactive hyperplasia in any of the salivary glands following the period of lysine-deficiency.

The lysine-deficient Group A revealed changes in bones and viscera similar to those described in the 30-day deficient animals. Groups E, F, and G were normal, but the best development was found in Group G, the controls fed ad libitum.

Study III.—In Figure 3 and Table 1 details are indicated concerning the growth, mortality, and pathologic features of animals in Study III. The parotid, sublingual, and submaxillary glands and the tongue showed better development in Groups H and I than in the 60-day lysine-deficient Group A. There were no differences between Groups H and I except for an interesting finding in one animal of the latter group: an early ductal carcinoma of the parotid. The bones and viscera of animals in Groups H and I were normal, but development was somewhat better in Group I. Both groups were better developed than in the 60-day lysine-deficient Group A.

Saliva.—Nitrogen and protein values were within the same range as in Study I. Total tyrosine was 15.4 mg. per cent, and the total "tryptophan" was 18.8 and 19.8 mg. per cent for the two pooled samples analyzed in Study III (Table 2).

Dental caries.—Rats in Group I (diet 636-1) had fewer carious teeth (1.89 versus 2.84) and a lower severity score (4.00 versus 6.26) than their pair-mates receiving diet 636. These differences have borderline statistical significance. No caries was found in the animals on diet LA for 60 days, Group A.

DISCUSSION

In these experiments lysine deficiency caused emaciation and serious impairment of general body growth, including the development of the salivary glands. The latter differed, in a quantitative manner only, on both gross and cellular levels. Although previously this diet had proved cariogenic after 60 days,[1] no caries was observed in the rats available for caries diagnosis after 60 days on experiment, Group A. These rats were individually housed, whereas dual housing, as in our previous study,[2] has usually proved effective for caries production in rats.

Glossitis was more common and more severe in animals deprived of lysine for 60 days than in those deprived for 30 days. Lysine deficiency lasting 60 days versus 30 days also appeared to cause an increased incidence of "spontaneous" pathology. Of these lesions, pneumonitis was the most frequent finding. However, control rats at 60 days also revealed a somewhat higher incidence of focal inflammation than at 30 days. Thus, despite the poor general condition and higher mortality of lysine-deficient rats, they were apparently no more susceptible to such lesions than were the controls.

The observations on the bones and viscera were similar to other reports[7-9] and provided no evidence of a specific effect of lysine deficiency. The bones of the lysine-limited rats, Group H, were reduced in size. Control rats receiving adequate lysine developed somewhat thicker, compact cortical and trabecular bone.

That salivary secretion plays a role in the complex etiology of dental caries is generally recognized. Recently, desalivation studies[16] indicated that a normal flow of saliva during eruption is of critical importance in enamel maturation and resistance to caries in the rat. In this study, lysine deficiency did not affect the total nitrogen, protein, tyrosine, and "tryptophan" of rats' saliva. Previously, the lysine content of rats' parotid saliva was found not to be affected by the lysine content of the diet.[17]

The mean rat salivary protein concentration of 0.76 per cent was in agreement with the results of others[18, 19] and exceeded the level reported for human mixed saliva.[20] Rat saliva also contained more tyrosine—i.e., 15.4 mg. per cent—than did mixed human saliva—10.7 mg. per cent.[21] Markedly more Ehrlich-positive material, indicated here as "tryptophan,"[15] was present in rat saliva—i.e., 18.8 and 19.8 mg. per cent—than in human mixed saliva—i.e., 2.8 mg. per cent,[21] 2.2 mg. per cent,[22] and 2.3 mg. per cent.[23]

No evidence has been obtained, therefore, in this or in previous studies to suggest any modification of the physiology of the salivary glands or the salivary secretion, concomitant with a lysine deficiency. In certain instances, however, as noted in this experiment and in previous studies, experimental dental caries was associated with lysine-deficient diets. A failure in rate of growth was observed, as in numerous previous studies with diets deficient in lysine or other essential amino acids.

SUMMARY

1. A study was made of young white rats given a lysine-deficient, zein-base diet, as well as a lysine-limited diet containing autoclaved skim-milk powder as the source of protein.

2. Impaired growth on this diet was corrected by a lysine supplement. Dental caries resulted on the skim-milk-powder diet. This diet was less cariogenic with a lysine supplement.

3. Salivary gland and tongue development on both gross and cellular levels was impaired by severe lysine deficiency, but not by the lysine-limited diet. Lysine deficiency produced no other distinctive gross or microscopic changes in these organs.

4. No changes were found in the total nitrogen, protein, tyrosine, and "tryptophan" content of the saliva due to inadequate lysine.

5. Although the mortality rate in 60-day lysine-deficient rats was increased, the incidence of "spontaneous" lesions, mainly pneumonitis, was no greater and apparently not a factor.

6. Repletion of animals with 1 per cent lysine daily after being subject to a deficient diet for 30 days produced no gross or microscopic changes in the salivary glands or tongue.

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The Fractionation of Human Tooth Enamel on the Basis of Density

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The hard outer layer of the tooth, or the enamel, has been intensively studied for many years. It is by far the hardest substance in the body and is composed primarily of inorganic salts. The enamel is about 98 per cent inorganic material, the remainder being water and organic substances. Both the inorganic and the organic constituents have been studied rather intensively,[1–12] but as yet there is no agreement concerning the exact composition of either.

The inorganic phase is an impure hydroxyapatite. The impurities consist of appreciable quantities of every inorganic ion found in the blood plasma. Some may actually be part of the crystal apatite lattice, while others may be adsorbed or in some way bound to the interface of the crystals. It is quite apparent that some of the ions, such as magnesium, may enter the crystal lattice in place of the calcium and that a flouride ion may replace a hydroxy ion, but there is considerable confusion concerning the position of such ions as carbonate, sodium, chloride, and others.

A microscopic study of the enamel indicates that it, in general, consists of bundles of rods usually pointing toward the pulp. Surrounding each rod or prism is the interprismatic substance. In the normal carious lesion this interprismatic substance is dissolved, in many cases leaving the rods without support.

When a sound central incisor is examined by means of an electron microscope, the surface often appears gnarled and quite rough (Figs. 1 and 2). However, if the surface is flattened by slight grinding, it may then be polished to a high luster with levigated aluminum oxide (Fig. 3). Replicas of this surface appear to be completely lacking in structural details. They give no indication of crystalline structure or of rods and interprismatic substance. If the smooth tooth surface, however, is polished with a coarser abrasive such as calcium carbonate, the smoothness disappears, and the outline of the enamel rods and interprismatic substance may be clearly observed (Fig. 4). It appears that the rods do not wear so readily as does the interprismatic substance, and hence the surface takes on the regular appearance of slightly elevated rod ends. Even though the interprismatic substance is clearly visible, no amorphous organic material can be observed. If either the highly polished surface, the raw tooth surface, or the calcium carbonate–polished surface is etched with acid such as 1 N hydrochloric acid for 5 seconds, the structure becomes clearly visible. The rods appear to be composed chiefly of highly compact crystals of inorganic material. The rods seem to dissolve more

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readily than the interprismatic substance, leaving ridges, partially crystalline and partially amorphous material, indicating considerable organic substance. It is quite possible that during the etching action, as soon as the organic material is liberated from the salt, it swells or hydrates, leaving the raised ridges of interprismatic substance (Fig. 5). The organic material appears to be concentrated around the periphery of each rod, and we may assume it to be impregnated with the same type of crystal or inorganic substances that appear within the rod. Because of the differences in solubility, there is considerable speculation concerning the chemical composition of the rod material and the interprismatic substance.

Insofar as the structures are extremely small, there have been no attempts to sep-

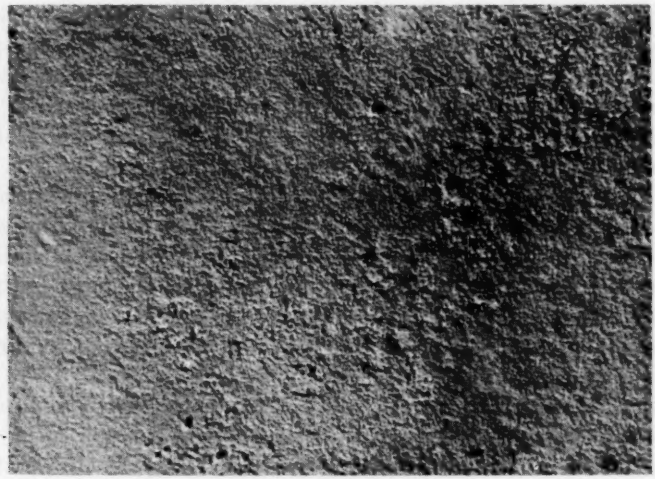

FIG. 1.—Human enamel. Clean, untreated surface. ×10,000

FIG. 2.—Human enamel. Clean, untreated surface. ×10,000

arate the rod substances from the interprismatic substances. In view of the fact that the periphery of the rods is so highly impregnated with organic material, it was thought that perhaps a separation could be made on the basis of density. The rod substances, having so little organic material, should be heavier than the substance that surrounds it. In view of this, attempts were made to separate the two on the basis of density.

EXPERIMENTAL METHODS

The surface of the enamel was cleaned and polished by means of an air-abrasive machine. The teeth were then split, after which the entire contents of the enamel shell

FIG. 3.—Human enamel. Highly polished with Al_2O_3. ×10,000

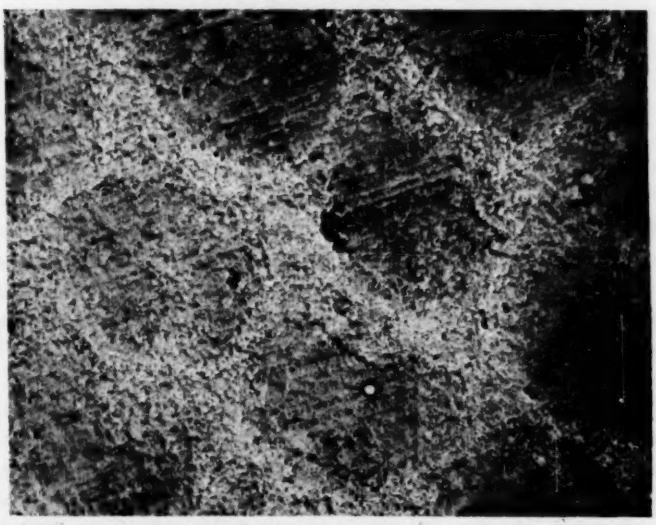

FIG. 4.—Human enamel, highly polished with levigated alumina, after which the surface was buffed with Sturges "Heavy" chalk. ×10,000.

was removed by means of burrs, diamond stones, and the air-abrasive. The entire occlusal surface was discarded because of the spindles of dentin protruding into the enamel. The enamel chips were then washed with water and inspected under a microscope for any residual dentin that might be present. If any dentin could be found by means of visual examination, the particles were rejected and re-worked. Only the pure, uncontaminated enamel was taken. It is quite probable that a number of lamellae or spindles of dentin were missed, but the material utilized was as pure enamel as could be obtained by physical means.

The enamel was then powdered in a hammer mill. This procedure contaminated the enamel with iron. Some difficulty was experienced in removing the iron. It was found that most of the iron could be removed by rapidly agitating the enamel particle in a water slurry, after which the enamel particles were decanted, leaving the iron particles

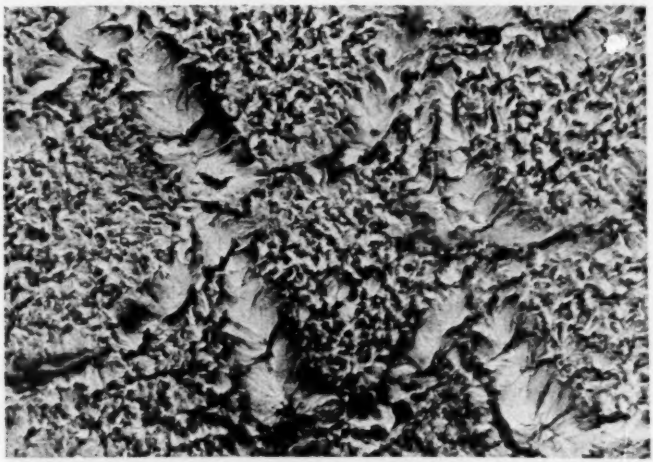

FIG. 5.—Human enamel. Buffed with $CaCO_3$ and etched 5 sec. with 0.1 N HCl. ×10,000

in the vessel. The rest was removed by means of a magnet, even though some enamel particles in which the iron was occluded were removed in the process. The enamel powder was then air-dried and separated into fractions according to the method of Armstrong,[13] using a bromoform-alcohol mixture, density 2.50. The light material was segregated from the heavy material, and each fraction was again subjected to further pulverizing and separation. It was found that this method did not produce particles fine enough to enable a separation with high organic content. For this reason another method of pulverization was devised. It was found that if the finely powdered, washed enamel from the hammer mill was allowed to pass into an air stream at 75 lb. pressure/square inch and forcibly blown through an orifice, it could be allowed to impinge upon a silicon carbide crystal with sufficient force to fracture the fine particles. The speed of the air stream was approximately 20,000 cm/sec. These particles would either settle out in the bottom of the container or float in the air stream, where they could be collected (Fig. 6). When these two fractions were successively analyzed and subjected to further pulverization, it was found that the organic content of the light particles increased, while that of the heavy particles decreased. This process was re-

peated until the organic content of the light material approached a constant value. Each fraction was analyzed for organic material according to the dichromate method of Johnson[14]—sodium and potassium by means of a flame photometer,[15, 16] calcium by titration of the oxalate,[17] phosphorus[18] and magnesium[19] by means of appropriate colorimetric methods. Carbon dioxide was measured manometrically in a Warburg vessel.[20]

At appropriate intervals, samples of the light and heavy materials were viewed under the electron microscope in order to determine particle size, after which they were slightly etched with lactate buffer, pH 4.5, in order to liberate any organic material present. The etched samples were again viewed in the electron microscope. Samples of the lighter and of the heavier fractions were sent to Dr. O. R. Trautz for examination with X-ray diffraction methods.

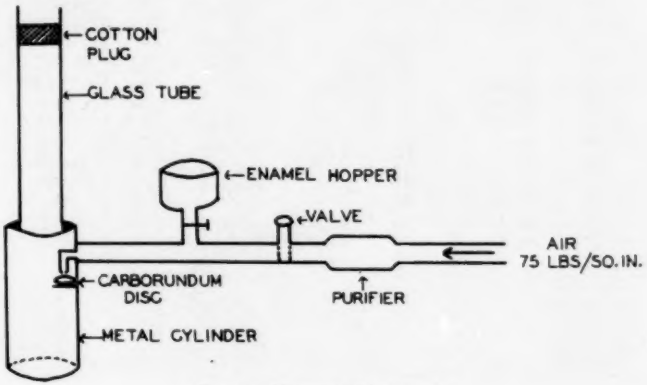

FIG. 6.—Enamel disintegrater

RESULTS

It was found that, as the fractions were progressively separated, the heavier particles decreased in organic content and the lighter fractions increased in organic content. The decrease in organic content progressed from 0.53 to 0.27 per cent. The increase in organic content of the lighter fraction approaches a limiting value of 2.3 per cent.

As the organic content decreased, there was a corresponding increase in each inorganic constituent. In the heavier fractions the Ca/PO_4 approached a value of 2.02. In the lighter fractions the ratio decreased to 2.00. If we assume that all the phosphorus is bound as the hydrated orthophosphate or apatite and that the carbon dioxide is all bound as the carbonate, the analysis as found accounts for 99.11 per cent of the total constituents in the unfractionated enamel. On the same basis, 101.38 per cent is accounted for in the heavy fraction, and 93.24 is accounted for in the light fraction. Because of the assumptions necessary for the calculation, it is questionable that any significance can be attached to these differences. If the assumptions are valid, however, it may well be that the light fraction is more hydrated than the heavy fraction or that there is a higher concentration of ions not determined by our analysis in the lighter fraction. The analytical data are given in Tables 1 and 2.

The X-ray diffraction pattern obtained in a Norelco diffractometer and interpreted by Trautz are essentially the same as those obtained from unfractionated enamel

samples. In particular, no other diffraction lines than the usual apatite lines were observed. The diffraction peaks of the lighter fraction were a little lower and slightly broader than those of the heavier fraction, indicating, if anything, a somewhat smaller average crystallite size in the lighter fraction.

The electron micrographs indicated that, by the use of the air-blast technique, particles of 0.1–6.0 μ were obtained. The particle appeared crystalline. When the particles were etched for 5 seconds with 0.1 N lactate buffer, pH 4.5, there was a marked difference between the light and heavy particles. The heavy particles partially dis-

TABLE 1

ANALYSIS OF HEAVIER FRACTION (PER CENT BY WEIGHT)

Fraction	Organic	Ca	P	CO_2	Mg	Na	K	Ca/P
0.....	0.53	35.70	17.68	2.75	0.52	0.534	0.057	2.01
1.....	.49	35.82	17.78	2.78	.56	.557	.058	2.01
2.....	.48	35.86	17.82	2.79	.55	.568	.059	2.01
3.....	.42	36.04	17.84	2.84	.58	.572	.061	2.02
4.....	.39	36.10	17.87	2.86	.59	.578	.063	2.02
5.....	.38	36.14	17.88	2.88	.60	.580	.064	2.02
6.....	.35	36.22	17.91	2.92	.63	.588	.065	2.02
7.....	.32	36.30	17.96	2.96	.65	.590	.067	2.02
8.....	.28	36.42	18.00	2.99	.66	.592	.069	2.02
9.....	0.27	36.48	18.06	3.02	0.67	0.593	0.070	2.02

TABLE 2

ANALYSIS OF LIGHTER FRACTIONS

Fraction	Organic	Ca	P	CO_2	Mg	Na	K	Ca/P
0.....	0.53	35.70	17.68	2.75	0.52	0.534	0.57	2.01
1.....	0.62	35.58	17.82	2.68	.47	.545	.056	2.01
2.....	0.73	35.30	17.56	2.66	.45	.544	.055	2.01
3.....	0.92	35.12	17.44	2.63	.44	.542	.053	2.01
4.....	1.05	34.62	17.26	2.61	.43	.540	.052	2.00
5.....	1.44	34.12	16.94	2.49	.41	.536	.048	2.00
6.....	2.03	33.24	16.56	2.40	.38	.531	.043	2.00
7.....	2.20	33.04	16.48	2.37	.37	.529	.041	2.00
8.....	2.31	32.98	16.46	2.35	.36	.528	.040	2.00
9.....	2.30	32.97	16.46	2.34	0.35	0.528	0.040	2.00

solved, leaving a very slight ghost or residue (Figs. 7, 8). The residue of particles was not uniform, but the residue always appeared within the range of the original particle size. The light particles, however, appeared to leave a huge volume of residue, and in many cases it was in excess of the range of the original particle (Figs. 9, 10). This clearly indicated that the lighter particles contained considerable organic material that swelled or hydrated on treatment with weak acid.

DISCUSSION

On the basis of the results obtained, there is no doubt that enamel may be fractionated on the basis of density. Whether or not this represents a separation of inter-

prismatic and rod substance is not certain but is likely. In view of the crystalline appearance of the particles obtained, it is very unlikely that those particles in the light fraction are composed of lamellae. By the same token, it is quite probable that the light fraction is from the periphery of the rod or from the interprismatic portions of the enamel. The material from the light fractions also behaves toward acids as does the interprismatic material. The organic portion, upon being freed from the inorganic, will swell and become much larger than the original crystalline material.

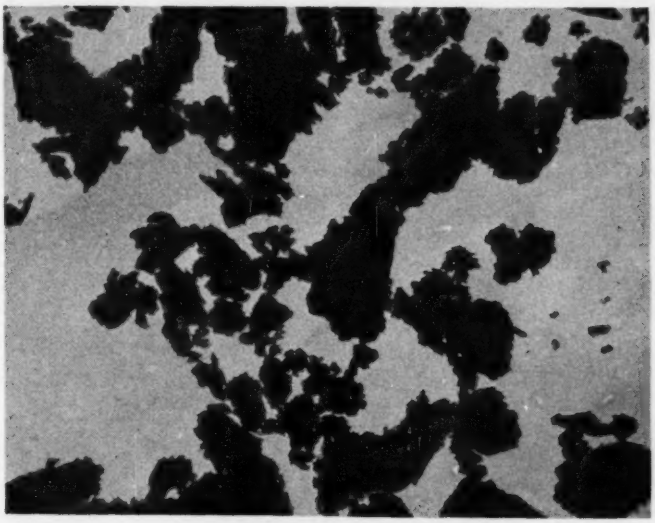

FIG. 7.—Heavy fraction. ×24,500

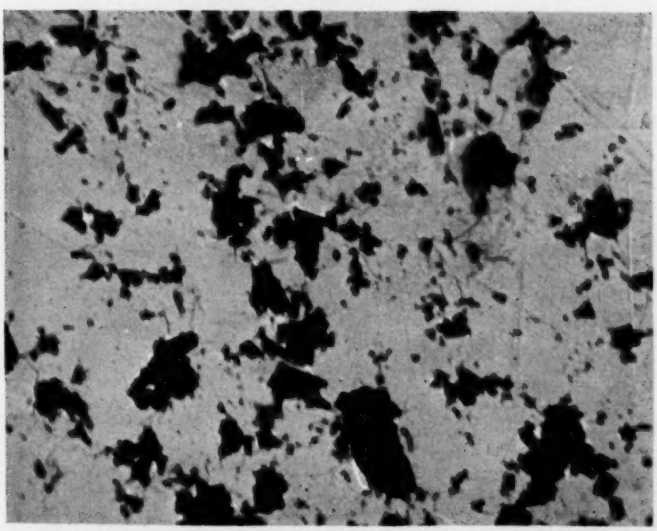

FIG. 8.—Heavy fraction. Etched 5 sec. with 0.1 N. lactate buffer, pH 4.5. ×24,500

The heavy fraction, on the other hand, has very little organic substance present. The electron-microscope studies indicate that it behaves toward acid in the same manner as do the enamel rods.

It was hoped that an analysis of the various fractions would show some trend toward differences in inorganic composition. Most of the changes involved, however, can be explained on the basis of the changes in organic content. The similarity between the X-ray patterns, together with the results of the chemical analyses, indicate that the

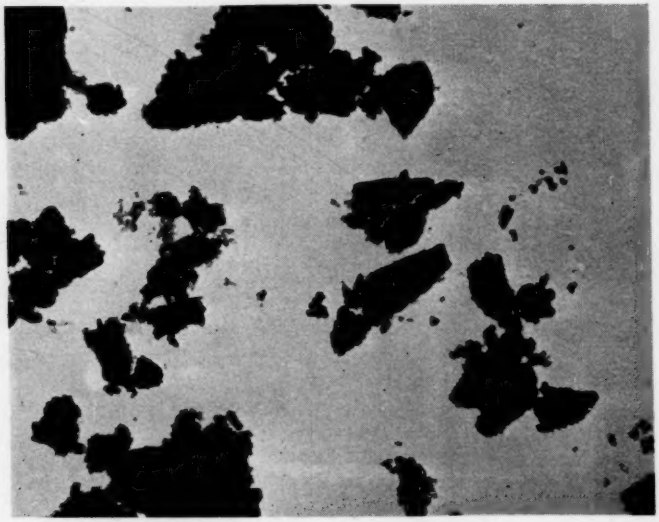

FIG. 9.—Light fraction. ×24,500

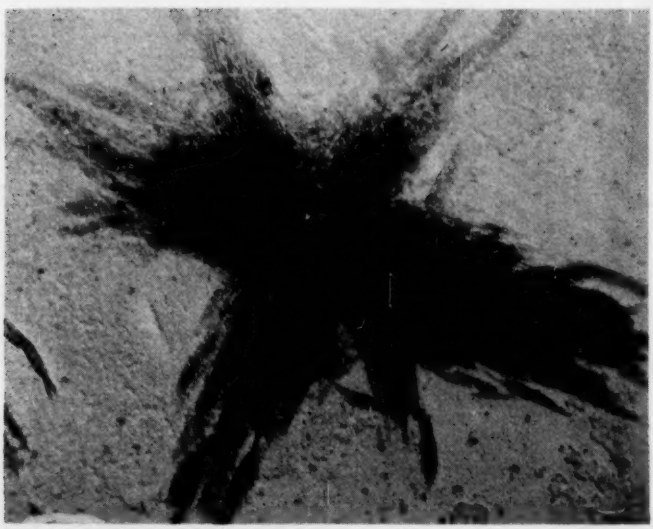

FIG. 10.—Light fraction. Etched 5 sec. with 0.1 N lactate buffer, pH 4.5. ×24,500

inorganic phase of the light fraction (probably the interprismatic substance) has a composition similar to that of the heavy fraction (probably the prism core).

SUMMARY

Human tooth enamel was finely divided by forcibly ejecting it against a silicon carbide crystal. The finely powdered enamel was then separated on the basis of density and analyzed for organic material, calcium, phosphorus, carbon dioxide, magnesium, sodium, and potassium. The more dense fraction of enamel contained 0.27 per cent organic material, while the light contained 2.3 per cent organic material. As the fractions became more dense, the calcium, phosphorus, carbon dioxide, magnesium, sodium, and potassium increased. The lighter fraction contained a lesser concentration of these inorganic ions. It is thought that the heavier fraction came primarily from the enamel rods, while the lighter fraction came from the interprismatic substance.

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Possible Role of Bacterial Hyaluronidase in Incipient Carious Lesions

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Previous work has shown that a number of pathological changes in the tooth structure precede actual cavitation.[1] Among these changes are a marked increase in permeability, an increase in reducing agents, and a possible depolymerization of the mucopolysaccharides. Such changes are typical of those produced by hyaluronidase.[2]

It was the purpose of this study to investigate the possible role of bacterial hyaluronidase in the pathogenesis of the incipient pathogenesis of carious lesions.

MATERIALS AND METHODS

Seventy Osborn-Mendel rats from eight litters were divided into two groups at 18 days of age. Thirty animals were placed on the cariogenic diet of the following composition: casein 24, sucrose 65, salts U.S.P. XIV 4, corn oil 5, dehydrated liver 4, vitamin mix 2, choline 0.2 parts. The forty remaining animals received the same diet, to which had been added 1 per cent by weight of an antihyaluronidase made by the method of Rogers and Spensley.[3] This material is a polyanionic inhibitor of hyaluronidase and apparently works by competing with the natural substrate for the hyaluronidase that may be present. All animals were sacrificed at 28 days of age, and the teeth were examined histochemically by the method previously described.[1]

TABLE 1

NUMBER OF INCIPIENT CARIOUS LESIONS FOUND IN ANIMALS RECEIVING ANTIHYALURONIDASE FROM 18 TO 28 DAYS OF AGE COMPARED WITH CONTROLS RECEIVING NONE

Diet	No. Animals	No. Incipient Lesions	Average
Cariogenic diet, 18–28 days	30	133	4.09
Cariogenic diet plus antihyaluronidase, 18–28 days	31	25	0.8

RESULTS

Nine animals receiving the antihyaluronidase died before the end of the experiment. The thirty-one surviving animals on the 1 per cent antihyaluronidase diet had an average of 0.8 incipient lesion per animal, whereas the thirty animals which did not

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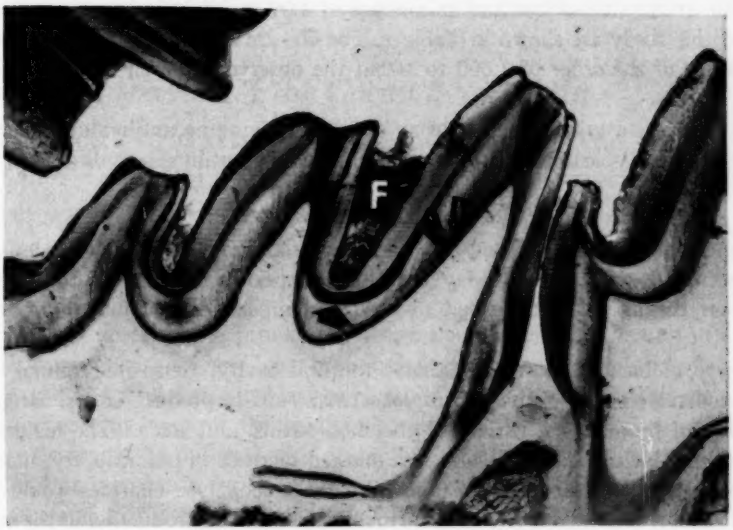

FIG. 1.—Section of teeth of 28-day-old animal receiving no antihyaluronidase in the diet. Note area of pathology in dentin. Section treated with ninhydrin to show presence of reactive protein. *D*, dentin; *E*, enamel; *F*, food.

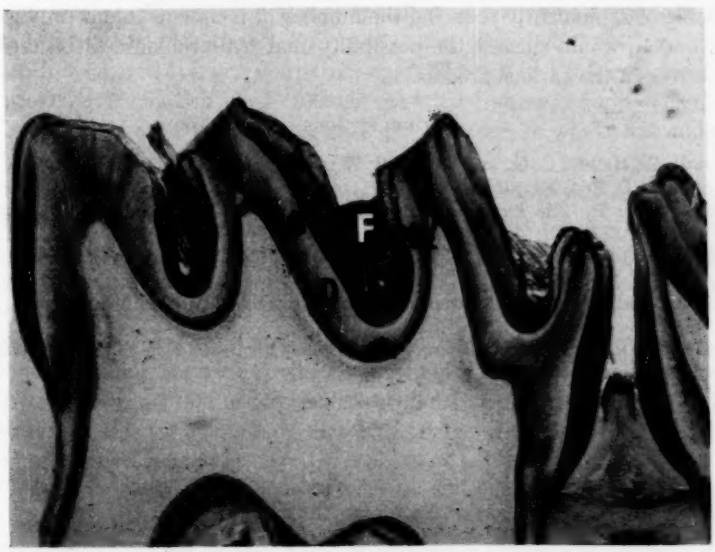

FIG. 2.—Sections of teeth of 28-day-old animal receiving 1 per cent antihyaluronidase in diet. Note absence of area of pathology in dentin. Section treated with ninhydrin to show presence of reactive protein. *D*, dentin; *E*, enamel; *F*, food.

receive the antihyaluronidase had an average of 4.09 incipient lesions per animal. The results of this study are shown in Table 1. The chi-square analysis of these data gives a probability of the order of 1,000 to 1 that the observed differences were not due to chance.

Figure 1 shows a typical section of an animal receiving no antihyaluronidase, while Figure 2 shows a typical section of an animal receiving antihyaluronidase.

DISCUSSION

The antihyaluronidase as used appeared to be toxic, as shown by the number of deaths occurring within the 10-day experimental period. In spite of the deleterious effect upon the animals, it seemed to reduce significantly the number of incipient lesions.

A variety of bacteria normally inhabit the oral cavity. Germ-free animal studies[4] have demonstrated the ability of *Streptococcus fecalis* to produce caries. Streptococci are capable of producing a variety of enzymes, toxins, and acids.[5] The histochemical analysis of the incipient lesion showing a marked increase in permeability, increase in reducing agents, and a possible breakdown of the mucopolysaccharides would suggest the possibility of bacterial hyaluronidase involvement in the production of the incipient lesion. The results of the present study would lend support to this concept.

CONCLUSIONS

1. An antihyaluronidase administered to animals on a cariogenic diet has been found capable of significantly reducing the number of incipient lesions produced.

2. This finding would suggest the possibility that hyaluronidase of bacterial origin may play a role in the carious process.

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Influence of the Ash of the Cacao Bean on the Cariogenicity of a High-Sucrose Diet

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A number of studies[1-8] have provided evidence that the mineral content of a diet may affect its cariogenicity. It may have been such investigations as these which led Rozeik, Cremer, and Hannover[9] to consider the possibility that the minerals in the cacao bean might account for some of the results obtained in the Vipeholm experiments.[10] In these experiments the subjects who ate 8 toffees daily between meals over a 2-year period had an increase in caries activity more than twice that of those who ate almost the same amount of sugar between meals in the form of milk chocolate.

In experiments by Rozeik, Cremer, and Hannover on sialoadenectomized white rats, there was considerably less dental caries in the animals fed a diet containing cacao-bean ash than in those on the control diet. These observations led the authors to conclude that the ash of the cacao bean is caries-inhibitory. It should be noted, however, that there were several differences in the composition of the control and test diets, apart from the ash content of the latter. The control diet consisted of 200 gm. barley groats, 100 gm. fish meal, and 100 gm. powdered sugar. The test diet was composed of 100 gm. barley groats, 100 gm. fish meal, 100 gm. powdered sugar, 66 gm. vitaminized margarine, 12 gm. casein, 12 gm. starch, and 6 gm. cellulose, to which was added 5.25 gm. of the cacao-bean ash.

One notable difference between the two diets was the addition of fat in the form of vitaminized margarine to the experimental diet, whereas the margarine was omitted from the control diet. Other investigators have found that when fat was added to a synthetic cariogenic diet, the diet was less conducive both to smooth-surface caries[2] and to fissure caries in the albino rat.[11]

In view of these considerations, two different experiments have been conducted in our laboratories to determine (1) whether the addition of cacao-bean ash to a synthetic high-sucrose cariogenic diet would render the diet less cariogenic and (2) whether supplementation of this ash-containing diet with fat in the form of vitaminized margarine would affect its cariogenicity.

EXPERIMENTAL METHODS AND RESULTS

In the first experiment twenty pairs of littermate albino rats of the Emory-Wistar strain were selected at weaning and sialoadenectomized by our usual procedure.[12] The

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control animals were fed a high-sucrose diet, the composition of which may be found in previous publications by Sognnaes and ourselves.[13-14] This diet, which we have designated as the "Harvard diet," has been used in a number of experiments in our laboratories and has been found to be highly cariogenic. The test animals were fed this same basic diet to which 2 gm. cacao-bean ash were added per 100-gm. diet. The cacao beans were ashed in a muffle furnace at 500°–600° C. The animals were allowed free access to food and water for 70 days and were sacrificed at the end of this feeding period. The mandibles and maxillae were removed and the teeth examined under a dissecting microscope and scored for dental caries according to the method routinely followed in our laboratory.[15]

The average number of carious lesions and the average caries score per animal are presented in Table 1. It is quite apparent that the cariogenicity of the Harvard high-sucrose diet was not affected by the addition of 2 gm. cacao-bean ash to 100-gm. diet.

The second experiment was conducted with the same procedure as the first. The basic diet was the same in the two experiments. In the second experiment 1.3 gm. cacao-

TABLE 1

DENTAL CARIES IN SIALOADENECTOMIZED RATS FED CARIOGENIC DIETS WITH AND WITHOUT ADDITION OF CACAO-BEAN ASH

Diet	No. of Animals	Av. No. of Carious Lesions	S.E.M.*	Av. Caries Score	S.E.M.
Test.....	20	25	±0.38	43	±3.0
Control....	20	24	±0.39	41	±1.9

* S.E.M. is the standard error of the mean.

bean ash were added to 100 gm. of both control and test diets. No additional fat was added to the control diet, whereas 20 gm. vitaminized margarine were incorporated in each 100 gm. of the experimental diet. In this experiment only 10 pairs of littermates were used. The curtailment in the number of animals and the smaller amount of cacao-bean ash added, as compared with the first experiment, were necessitated by the diminishing supply of cacao-bean ash after completion of the first experiment. It was thought undesirable to add to the supply that we had on hand by placing another order for cacao beans because of the probability that they would have been grown in a different geographical area. However, the ash concentration at this level was approximately the same as in the experiments of Rozeik, Cremer, and Hannover. The animals were sacrificed at the conclusion of a 60-day feeding period, and the teeth were examined and scored for caries by our usual procedure. The results of the experiment are given in Table 2. The shorter duration of the second experiment probably accounts for the lower caries score than in the first experiment.

Each animal on the test diet had, on the average, 4 carious lesions less than its control. The average caries score was 21, as compared with the score of 31 for the controls. These differences in the number of carious lesions and in the caries scores were both statistically significant. It may therefore be concluded that the addition of margarine

to the diet containing cacao-bean ash reduced its cariogenicity. In the light of these results, the lowered cariogenicity of the ash-containing diet in the experiments of Rozeik, Cremer, and Hannover would appear to us to have been brought about most likely by the addition of fat to the diet and possibly to other differences in the test and control diets rather than to the cacao-bean ash itself.

We could not, however, overlook the possibility of a difference in the composition of the cacao-bean ash used in the European and the American laboratories which could conceivably have resulted in a reduction in caries when one ash was added to the diet but not when the other ash was added. The cacao beans used in our experiment were purchased from a New York broker and in all probability were grown in a different area than the cacao beans used in the experiments of Rozeik, Cremer, and Hannover. In this connection one recalls the experiments[16-17] in which the incidence of dental caries in rats was affected in a different degree by the same kinds of foodstuffs produced in different soil areas. We have accordingly analyzed the dried ash of the cacao beans used in our experiments. Comparison of these analytical data with those obtained by Rozeik, Cremer, and Hannover can be made by referring to Table 3. As a matter of

TABLE 2

EFFECT OF ADDING MARGARINE TO A CARIOGENIC DIET CONTAINING CACAO-BEAN ASH

Diet	No. of Rats	Margarine Added	Av. No. Carious Lesions	S.E.M.*	C.R.†	Av. Caries Score	S.E.M.	C.R.
Control	10	None	23	±0.6	5.5	31	±2.1	4.3
Test	10	20 gm/100 gm	19	±0.4		21	±1.0	

* Standard error of the mean.

† The critical ratio (C.R.) is the ratio of the difference between two means to the standard error of the difference between the means. When the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when 2.0–2.9, the difference is of borderline significance; when 3.0 or more, the difference is highly significant (J. M. Dunning, *J. D. Res.*, **29**:541, 1950).

TABLE 3

ANALYTICAL DATA ON CACAO-BEAN ASH

	Ash Used in German Experiments (Per Cent)	Ash Used in Present Experiments (Per Cent)	Ash of Beans Grown in Bahia* (Per Cent)	Ash of Beans Grown in Haiti* (Per Cent)
K_2O	34.30	38.00	37.96	30.41
Na_2O	1.50	0.17	1.50	2.63
CaO	4.04	3.20	3.60	5.18
MgO	16.00	16.00	15.65	14.15
Fe_2O_3	0.31	0.31	0.60	1.93
Al_2O_3	0.33	0.89		
SiO_2	0.17	2.59	1.60	7.34
P_2O_5	32.00	27.70	32.60	27.65
SO_3	3.10	6.18	2.57	2.66
Cl	0.34	0.21	0.20	0.48
CO_2	5.80	1.06	3.72	7.57

* Values obtained from R. Whymper: *Cocoa and Chocolate* (2d ed.; Philadelphia: P. Blakiston Son & Co., 1921), Table XXVII, p. 148.

interest, we have included in the table data on the ash of cacao beans grown in Brazil (Bahia) and Haiti.

While there are some differences in the composition of the cacao-bean ash used in the experiments that were conducted in the German and American laboratories, which at first glance might appear to be relatively large, actually the difference in the total concentration of any element in the diet, as a result of adding one or the other cacao-bean ash to the diet, would be inappreciable. The percentage of sodium and phosphorus, for example, is considerably higher in one ash than in the other. However, if 1.3 gm. ash obtained from the two lots of cacao beans were added to diets of the same composition, the difference in the sodium and phosphorus content would amount to approximately only 0.01 and 0.02 per cent, respectively. It is highly improbable that these slight differences in the mineral composition of the diets which would be brought about by addition of the one instead of the other ash would affect its cariogenicity. In experiments previously reported from our laboratories,[18] it has been found that the caries conduciveness of our high-sucrose diet was not affected by an increase in the sodium content from 0.44 to 0.56 per cent. We are therefore led to conclude that the difference in the results of our experiments and those of Rozeik, Cremer, and Hannover cannot in any way be accounted for by a difference in the mineral content of the cacao-bean ash.

SUMMARY

The addition of the cacao-bean ash to a high-sucrose diet fed to albino rats did not reduce the cariogenicity of the diet.

It would therefore appear unlikely that the minerals in chocolate can account for the lesser increase in dental caries in the subjects of the Vipeholm experiments who ate chocolate between meals than in those who ate the same amount of sugar in the form of toffees.

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Comparative Histology of Age Changes in Oral Tissues of Rat, Hamster, and Monkey

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In recent years there has been an increased awareness of the importance of those tissue changes which result from age. The numerous changes in the oral cavity of the rat include downgrowth of the epithelial attachment, increased inflammation, distal drift of the molars, loss of interproximal bone, continuous cemental deposition, and decreased width of the periodontal membrane.[1–5] In a recent study on aging in mice,[6] pocket formation, associated with accumulation of debris, was found to occur around the maxillary molars, but no downgrowth of the epithelial attachment was observed in the mandible. In the same study it was found that the non-trabecular bone of the 10-month-old animal became trabeculated and the marrow spaces filled with connective tissue by 24 months of age. Atrophy of the alveolar crest was not observed, although food impaction and inflammation increased with age. Studies on hamsters have disclosed an increased incidence of periodontal disease with increasing age.[7-9] The disease process is characterized by accumulations of crevicular debris, inflammation, pocket formation, and downgrowth of the epithelial attachment. Using the smear technique in the study of the oral tissues of human patients, both increased keratinization[10-12] and lack of change in the exfoliative cytology have been reported.[13] In another investigation on biopsied human gingiva, increased coarseness of the connective tissue fibers and decreased cellularity have been described.[14] No cytologic change was found in the epithelium or in the degree of cornification.[15] Atrophy of the prickle-cell layer of the oral mucous membrane, which can be modified by estradiol therapy, has been observed in postmenopausal patients.[16] In some aged individuals an atrophy of the oral mucous membrane, characterized by a loss of keratinization, loss of elasticity, and increased friability, has been described.[17, 18] In addition, a narrowing of the human periodontal membrane[19] and a decrease in its cellular elements[20] have been reported in the literature.

In the present study, an attempt has been made to compare the aging patterns of two commonly used laboratory animals—the rat and the hamster—with those of a primate —the monkey. The studies on the rodents enabled us to observe the changes in life-span in a short interval and compare them with those in a monkey. Presumably, the monkey may more closely simulate those changes which may be expected in the human.

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MATERIALS AND METHODS

The animals used in this study consisted of 18 hamsters and 18 Long-Evans rats. They were sacrificed by intraperitoneal injections of Nembutal at 20 days of age, 140–200 days of age, and 450–650 days of age. The jaws of three monkeys were also studied. The age of the monkeys was determined by X-ray examination of the stage of eruption of teeth and by the estimates from the commercial suppliers. They were approximately 2, 6, and 9 years of age. The animals were fed Purina laboratory chow ad libitum. The jaws of the rats and hamsters were fixed in Bouin's fluid and 10 per cent formalin, decalcified in 5 per cent nitric acid, imbedded in paraffin, and sectioned at 10μ. The monkey jaws were treated the same way except that celloidin imbedding was utilized. The sections were stained with H and E Mallory's connective tissue stain, Van Gieson's picro-fuchsin, and Lillie's PAS.

TABLE 1

DOWNGROWTH OF EPITHELIAL ATTACHMENT AS MEASURED FROM CEMENTO-ENAMEL JUNCTION TO LOWEST POINT ATTACHMENT (MM.)

	Immature	200 Days	600 Days	Root Length of Oldest
Hamster	0	0.03–0.12	0.24–0.55	3.5
Rat	0	0–0.08	0.06–0.25	3.5
Monkey	2 year	6 year	9 year	.....
Deciduous	0.12	.....	.....	.....
Permanent	0	0.09	0.16–0.3	10

RESULTS

Gingiva and epithelial attachment.—Examination of the gingiva of the rats revealed no alteration in total thickness of the epithelium, stratum granulosum, or keratinization, although the gingival epithelium of two of the oldest animals, over 600 days in age, was found to be decreased in thickness. The greatest change in the gingiva was in the connective tissue, which was less cellular and more fibrous with increasing age.

Alteration in position of the epithelial attachment was calculated for molar teeth by measuring from the cemento-enamel junction to the most apical point of attachment by means of a calibrated ocular micrometer. The attachment was found to be at a more apical level, around all the teeth of all the older animals. As shown in Table 1, the downgrowth observed in the oldest monkey and rats was approximately the same, 0.16–0.3 mm. for the former and 0.06–0.25 mm. for the latter. The interval measured for the oldest hamsters exceeded that of the monkey or rats, being 0.24–0.55 mm., of which 0.1–0.2 mm. involved uncovered cementum. In hamsters the exposure of the cementum in the interproximal region was due to destruction of the interproximal papillae. No exposure of cementum was ever observed around the teeth of the monkeys or rats, despite the downgrowth of the epithelial attachment.

The length of the roots was measured from the cemento-enamel junction to the apex and was found to be 10 mm. for the oldest monkey and 3.5 mm. for the oldest rats and hamsters. Thus, in terms of percentage of the root lengths, the degree of downgrowth is

15 per cent for the 600-day hamsters, 7 per cent for the 600-day rats, and 3 per cent for the 9-year-old monkey.

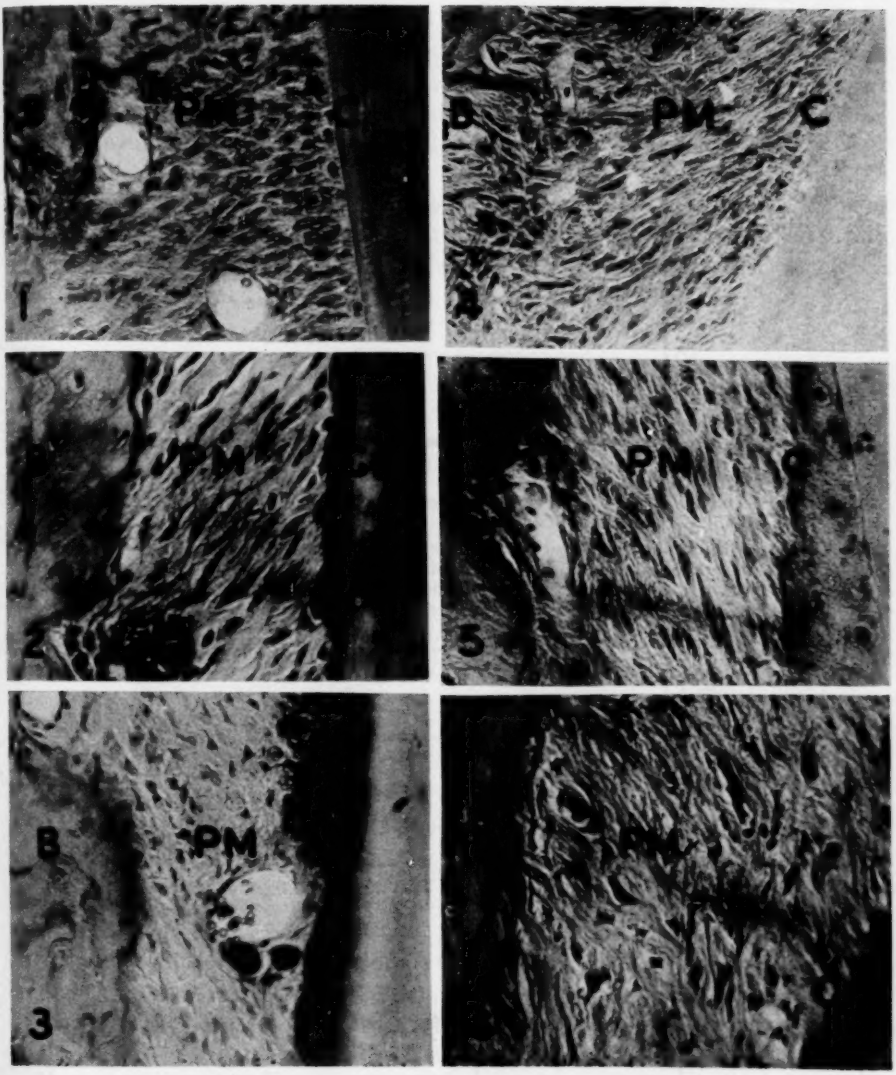
Periodontal membrane.—The periodontal membrane was examined for alterations in cellularity, connective tissue fibers, and mesiodistal width. The cellularity was measured in a standard manner for all the animals by counting the number of fibroblasts in 0.07 mm.^{2} areas with an ocular micrometer. The counts were made of sagittal sections of the molar roots, keeping the borders of the counting area a sufficient distance from the surfaces of the cementum and bone to avoid including cementoblasts or osteoblasts in the counts. Successive squares were counted, beginning at the alveolar crest and progressing down to the apex of the root, skipping only those areas where blood vessels intervened.

The periodontal membrane of the youngest rats and hamsters is extremely cellular and contains relatively few fibers (Figs. 1 and 4). Under low power of the microscope, it appears to be composed almost entirely of nuclei of fibroblasts, cementoblasts, and osteoblasts. It is slightly widened at the coronal end but is evenly contoured and maintains nearly a constant width to the apex of the root. At 200 days of age (Figs. 2 and 5), the cellularity of the periodontal membrane of the rats and hamsters is somewhat diminished, and the connective tissue fibers, particularly the alveolar crest and septal fibers, have thickened considerably, becoming tendinous in appearance. At 600 days of age (Figs. 3 and 6), the periodontal membrane of the rat and hamster has become irregular in shape, and there is considerable variation in the widths in different regions of the teeth. Unlike the younger animals, there are some very narrow areas in comparison with those which have remained wide. The decreased cellularity persists in older animals and in some areas is even more pronounced. Occasionally, cellular fields, similar in appearance to the periodontal membrane of the 20-day-old animals, can be found at 600 days of age. This is indicated in Figure 7, by the increased spread in total counts which occurs with increasing age. The most cellularity was usually found in the alveolar crest region.

The jaw of the 2-year-old monkey in the region where the first permanent molar has just erupted and the deciduous second molar is about to be shed was studied. The newly erupted permanent molar has a cellular, immature periodontal membrane, while, alongside, the relatively acellular and more fibrous periodontal membrane of the older deciduous tooth can be seen. In effect, the periodontal membrane of the newly erupted tooth is an area of local rejuvenescence. Some decreased cellularity was observed with increasing age in the periodontal membranes of the 6- and 9-year-old monkys, but it was much less than in the 600-day rat and hamster (Fig. 8).

The periodontal membrane of the monkey and hamster appeared more vascular externally than that of the rat at all ages, while the increased density of the connective tissue fibers was most marked in the rat. Histologically, the over-all pattern was similar for the same ages of the three animals studied.

Inflammation.—Inflammation was measured by counting the number of inflammatory cells in a 0.05-mm.^{2} area under medium power of the microscope. To standardize the procedure, all the counts were made just under the epithelium of the interproximal region of the molar. Complications arose when studying sections of hamster jaws, as the interproximal papillae were usually destroyed in all but the 20-day-old animals. Furthermore, in the hamster the bulk of the inflammatory cells apparently migrate



FIGS. 1–6.—FIG. 1: Periodontal membrane of 20-day-old hamster showing cellularity. *B*, alveolar bone; *PM*, periodontal membrane; *C*, cementum. (H & E ×390.) FIG. 2: Periodontal membrane of 200-day-old hamster. Note decreased cellularity. (H & E ×390.) FIG. 3: Periodontal membrane of 600-day-old hamster. Note further decrease in cellularity and irregularity of cementum. (H & E ×390.) FIG. 4: Periodontal membrane of 20-day-old rat showing cellularity. (H & E ×390.) FIG. 5: Periodontal membrane of 200-day-old rat showing decreased cellularity. (H & E ×390.) FIG. 6: Periodontal membrane of 600-day-old rat showing further decrease in cellularity and pyknotic-appearing nuclei. (H & E ×440.)

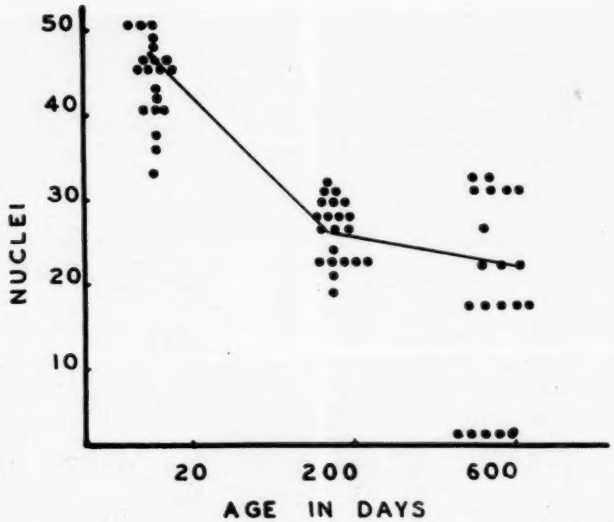


FIG. 7.—Nuclei of fibroblasts counted in successive 0.07 mm.^{2} areas of the periodontal membrane of the rat and the hamster. Since the fibroblastic counts for the two animals were identical, all the data are shown on this single graph. There is an abrupt decrease in concentration between 20 and 200 days of age and a more gradual decline and an increased spread between 200 and 600 days.

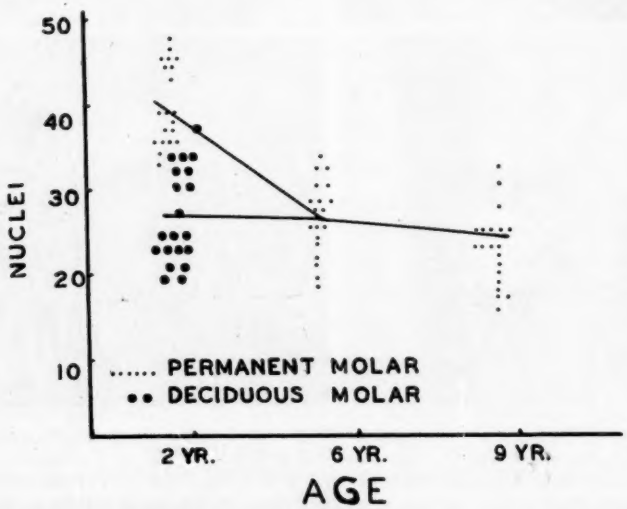


FIG. 8.—Nuclei of fibroblasts of periodontal membrane of 2-, 6-, and 9-year-old monkey. Note that the deciduous tooth of the 2-year-old monkey and the permanent tooth of the 9-year-old monkey have similar concentrations of nuclei.

through the epithelium and are found outside the gingiva. In order to take this into consideration, two counts were made for each hamster section, one just under the epithelium and one just superficial to the epithelium, both midway between the molars. A count of 60 cells completely filled the counting area and constituted the maximum inflammation. This concentration is equivalent to heavy inflammation in the classification used by Belting,[2] in which he placed inflammation in four categories from light to heavy.

As indicated in Figure 9, few inflammatory cells were visible in the papillae of immature rats, and only a slight increase occurred at 200 days of age. At 600 days, little additional inflammation was found in the rat (Fig. 12). In the immature hamster, as in

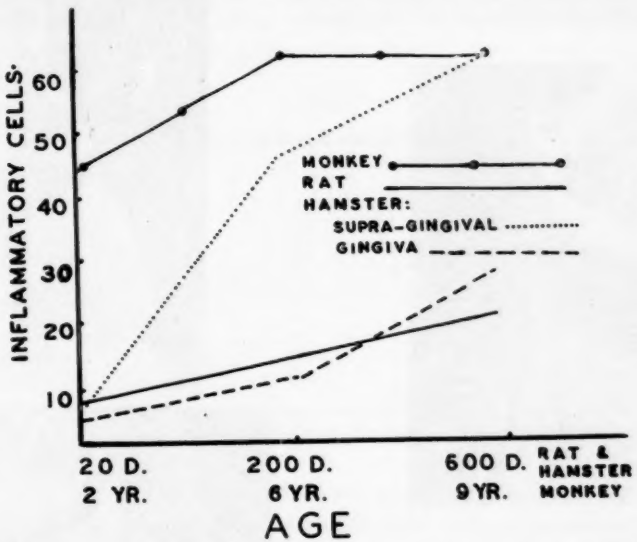
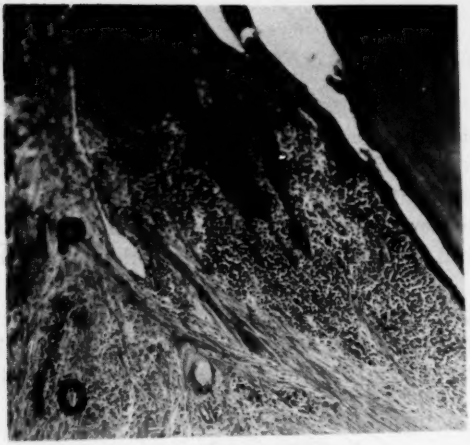


FIG. 9.—Inflammatory cells counted in 0.05 mm.^{2} area of interproximal papillae of rat, hamster, and monkey. Note that the most inflammation at any age is found in the monkey and that the greatest increase with age is in the hamster. Count at any age represents the average of 100 fields.

the immature rat, few inflammatory cells were seen. In the 200-day hamster, however, the inflammation was markedly increased, though mostly supra-epithelial. At 600 days of age in the hamster, the increase was still more marked, being almost entirely supra-epithelial (Fig. 11).

Much dense lymphocytic infiltration was observable in the gingiva of the 2-year-old monkey (Fig. 10), although externally the gingiva appeared normal. The inflammatory cells were concentrated just under the epithelium and extended 0.3–0.5 mm. below the most apical portion of the epithelial attachment. Similar inflammation was encountered in the 6- and 9-year-old monkeys.

The most marked increase in inflammation that could be associated with increasing age occurred in the hamster and appeared to parallel the increase in crevicular debris. The gingival crevices of the rats and monkeys appeared relatively free of debris at all ages. As shown in Figure 9, the heaviest inflammation at all ages was found to occur in



FIGS. 10–15.—FIG. 10: Section of an interproximal papilla in the molar region of a 2-year-old monkey. Note severe gingivitis. *D*, dentine; *IP*, interproximal papillae. (H & E ×90.) FIG. 11: Interproximal area of 600-day-old hamster. The papilla has been replaced by debris and inflammatory cells. (H & E ×90.) FIG. 12: Section through interproximal papilla of 600-day-old rat. There is little inflammation, and the form of the papilla is well preserved. (H & E ×90.) FIG. 13: Interradicular bone of recently erupted first mandibular molar of 2-year-old monkey. (H & E ×20.) FIG. 14: Interradicular bone of mandibular first molar of 6-year-old monkey. Note decreased density in apical region. (H & E ×20.) FIG. 15: Interradicular bone of mandibular first molar of 9-year-old monkey. Note further decreased density. (H & E ×25.)

the gingiva of the monkeys. The inflammation in the monkey did not appear to be associated with, or altered by, increasing age.

Cementum.—Estimates of the thickness of the cementum were made by comparing three measurements (Table 2). The first was made at right angles to the long axis of the root, about 0.05 mm. apical to the cemento-enamel junction. It was not made at the cemento-enamel junction, as, in many instances, the most coronal 0.05 mm. of cementum tapers almost to a point at the cemento-enamel junction. A second measurement was made in the same manner as the first, but midway down the length of the root. The third was made parallel to the long axis of the root from the end of the dentin to the root tip.

In the three animals studied, acellular cementum is formed first at the coronal end of the tooth. Soon afterward, cellular cementum is deposited in the apical region and adds to the length, as well as the width, of the roots.

TABLE 2*

THICKNESS OF CEMENTUM (MM.)

	CE Junction	MLR	Apex
Deciduous (2-yr. M.)	0.018	0.023	.....
Permanent (2-yr. M.)	.010	.010	.....
6-yr. M.	.02	.04	0.05–0.08
9-yr. M.	.028	.033	.8
200-day H.	.01	.01	.05
600-day H.	.01–0.02	.01–0.06	.5 – .6
200-day R.	.01	.01	.07
600-day R.	0.03	0.04	0.5 –0.6

* CE = cemento-enamel junction; MLR = midway length of root; M = monkey; H = hamster; R = rat.

At 200 days of age, the rat and hamster have a considerable amount of cellular cementum formed around the apexes of all the molar roots. The acellular cementum has increased in thickness both on the sides of the roots and in the interradicular area. At 600 days, the cementum of the hamster and rat has increased in thickness, but the pattern is irregular in the case of the hamster. As shown in Table 2, measurements made on the same side of the same root can vary from 0.01 to 0.06 mm. Numerous cementicles are visible in the hamster periodontal membrane at this age, and their formation may have caused the irregularities. In the bifurcation area and the epithelial attachment area of the 600-day hamster, the cementum deposition appears to have been disturbed, and the cementum seems to be disintegrating. This effect has apparently been caused by exposure to oral fluids due to epithelial attachment downgrowth.

Halfway down the root, the cemental thickness of the 600-day-old rat is 0.04 mm., which is about the average of the 0.01–0.06 mm. of the 600-day hamster. At this level, the cementum is laid down more evenly in the 600-day rats than in hamsters of the same age, and cementicles were rarely observed in the periodontal membrane of the rat.

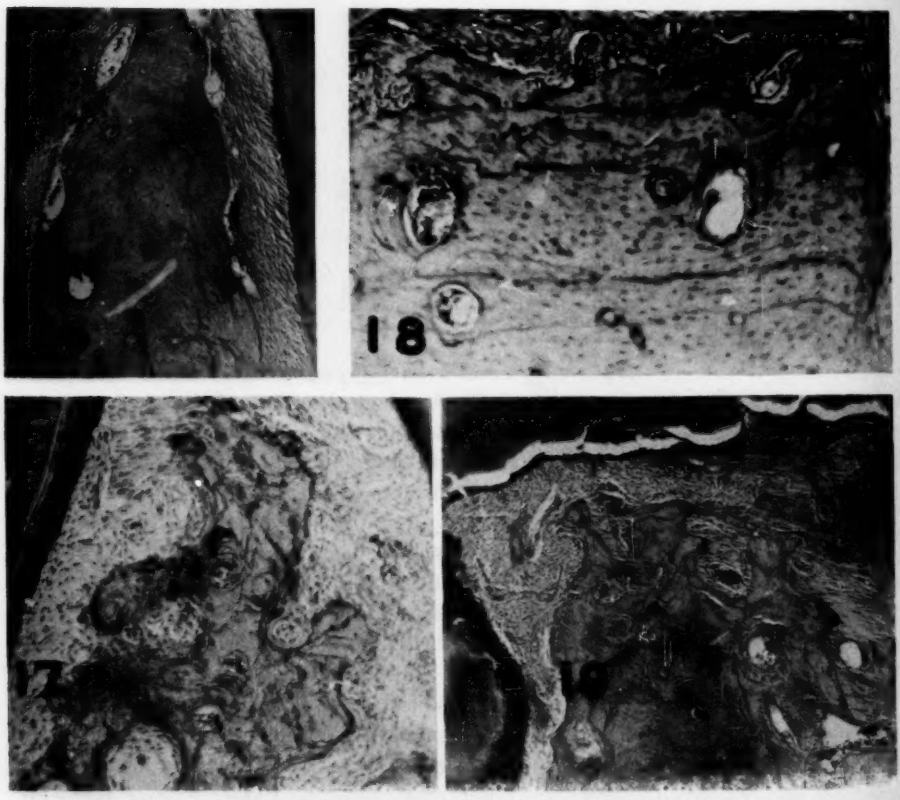
The molar region of the 2-year-old monkey with a mixed dentition was examined, and well-developed cellular and acellular cementum was observed on the deciduous molars, which were in the process of being shed. On the newly erupted permanent molar, a small amount of acellular cementum was present coronally, and cellular

cementum was just beginning to form at the apex. The cementum of the deciduous second molar was found to be thicker than that of the newly erupted permanent first molar. The corresponding tooth of the 6- and 9-year-old monkeys had progressively increased deposition.

As indicated in Table 2, the deposition of cementum increased with increasing age in a similar manner in the three animals studied.

In sections of all the oldest animals stained with H and E, the nuclei of the cementocytes took the basophilic stain and appeared intact, confirming the observations of Paynter in 1958.[21]

Alveolar bone.—Between 20 and 200 days of age the alveolar bone of rats and hamsters is transformed from an immature trabeculated bone to a dense, lamellated bone. There are fewer osteoblasts ond osteoclasts visible with increased age, and the marrow spaces are diminished. At 600 days of age, the bone of the rats and hamsters appears to undergo a fibrotic replacement (Figs. 16–19). Portions bordering the teeth



FIGS. 16–19.—FIG. 16: Section through interradicular bone of molar of 200-day-old rat. (H & E ×90.) FIG. 17: Section through interradicular bone of molar of 600-day-old rat. Note fibrotic replacement of periphery and marrow. (H & E ×130.) FIG. 18: Section through interradicular bone of molar of 200-day-old hamster. It is similar to that of 200-day-old rat. (H & E ×130.) FIG. 19: Section through interradicular bone of molar of 600-day-old hamster showing fibrotic replacement. (H & E ×90.)

have numerous fibrous lacunae, and the periphery or whole of many of the remaining marrow spaces has become filled with collagenous connective tissue. The fibrosis is most marked near the crowns of the teeth, while some intact marrow can be found around the apexes of the roots. At this age the bone takes on a contoured appearance due to the numerous irregular cement lines that are found. These lines are most prevalent in the aged rats, presumably because of the drift of the teeth. By 600 days, all or almost all the interproximal bone is resorbed in the rat as the interproximal spaces close. The alveolar bone of the 6-year-old monkey appeared less dense than that of the 200-day rat and hamster, particularly in the apical region. In the older monkeys, the marrow spaces appeared to increase in size and be replaced with adipose tissue. This alteration affected only the supporting bone but not the cortical bone or lamina dura, which remained intact at all ages (Figs. 13–15).

There was no measurable closure of interproximal space or resorption of interproximal bone in the molar region of the monkey or hamster at any age. A measurable lowering of the alveolar crest, as judged by its relation to the cemento-enamel junction, was found only in the aged hamsters.

DISCUSSION

In the rat a more apical epithelial attachment appears to be a part of physiologic aging. This downgrowth is necessary to preserve the clinical crown from shortening because of attrition and is perfectly compensated for by cementum deposition. In the hamster, judging by wear on the dentin, there is less attrition. If there is less occlusal wear on the hamster teeth, it is possible that there is less proximal wear too, and this could explain the lack of closure of the interproximal space. The downgrowth of the epithelial attachment in the hamster is so rapid that cementum deposition in the bifurcations is interfered with, and bifurcation involvements are frequent findings in old hamsters. It is possible that, on a more suitable diet, the hamster gingiva would more closely follow the pattern of the rat.

It is interesting to note that, in the presence of relatively intense gingival inflammation, there is no increased resorption of the alveolar crest in the monkey. In the rat little bone resorption takes place with little inflammation, and in the hamster a great deal of bone is resorbed, although the inflammation is largely supra-epithelial.

The periodontal membrane of the deciduous second molar of the 2-year-old monkey is relatively acellular in appearance. It lies adjacent to the cellular, immature periodontal membrane of the newly erupted first molar. This may indicate that the decreased cellularity found to occur with increasing age may be due, at least in part, to local factors rather than to aging per se of the entire organism. Kronfeld discusses several cases of teeth erupting in the mouths of elderly individuals. He does not describe the histology of these teeth, and it remains to be determined whether cellular, non-fibrous tissue would be found around them. Unerupted teeth in individuals of advanced age do undergo fibrosis.[22]

The marked increase in density and coarseness of the periodontal fibers may well reflect the increased thickness and length of collagen fibrils associated with aging which has been reported in electron-microscope studies (Gross, Keech).[23,24] There is evidence that the fibril has elastic properties which are lacking in the fiber (Randall).[25] This would seem to indicate that the older periodontal membrane is less elastic and

therefore more susceptible to the effects of occlusal trauma. Furthermore, it has been reported that old collagen is less susceptible to enzymatic digestion (Asboe).[26] This may be due to its increased density and difference in composition.

It should be noted that, with increasing age, there was an increase in variaton of tissue morphology. Although, as described, the tissue becomes less cellular and more fibrous in the aged animal, isolated areas which retained a "youthful" appearance could be found. Although the periodontal membrane becomes thinner, both thick, thin, and irregular-width periodontal membranes could be found in the older animals. By contrast, in youth all the tissues appear to have a more uniform morphology and structure.

SUMMARY

The patterns of aging of the oral tissues of the monkey, rat, and hamster were studied. The monkey was found to undergo the most severe gingival inflammation. The apical progress of the epithelial attachment was fastest in the hamster. The alveolar bone increases in density from immaturity to maturity and then decreases in density in the three animals studied.

Downgrowth of the epithelial attachment, continuous cementum deposition, fibrosis, decreased cellularity, and osteoporosis are consistent findings associated with aging.

It was noted that a characteristic of the aged animal was an increased variation in tissue morphology.

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Sibling Similarities in Dental Development

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Numerous aspects of the human dentition have been subjected to genetical analysis. Besides caries susceptibility, these include tooth size, tooth morphology, the time of eruption of the teeth, and, finally, the rate of development of the teeth within the jaws.

Tooth form does appear to be largely gene-determined, as studies of cusp pattern, crown morphology, and root shape suggest.[1,2] The size of the teeth may also be listed among those traits primarily under genetic control, even though twin concordance in crown diameters of the anterior teeth falls short of perfection.[3,4] For the timing of tooth eruption there are at least inferential data, derived from comparative studies, that point to some degree of genic control.[5,6] However, information on twin and sibling similarities in the rate of tooth formation and comparative racial data on tooth formation are almost completely lacking.[7]

We have, therefore, extended our earlier studies on sibling similarities in the *sequence* of calcification[7] to similar family-line analysis of similarities in the timing of tooth calcification and tooth movement. Our purpose has been to determine whether and to what extent tooth development shows evidence of genic control.

MATERIALS AND METHODS

The present investigation is based upon serial longitudinal oblique-jaw radiographs of 170 white Ohio-born siblings from 62 nuclear families, accounting for a maximum of 175 sibling pairings. Two sets of triplets were separately analyzed and tabulated.

From the serial radiographs available for each child, beginning with the first postnatal month, the age at attainment of three calcification stages and two stages of tooth movement was separately ascertained for $\overline{P}_1$, $\overline{P}_2$, $\overline{M}_1$, $\overline{M}_2$, and, where possible, for $\overline{M}_3$. The tooth calcification stages were (1) initial calcification, (2) beginning root formation, and (3) apical closure. The movement stages were, in turn, (1) alveolar eruption and (2) attainment of the occlusal level. A complete description of these stages, as defined in our studies, as well as a rationale for their use, has been published in this *Journal*.[8,9]

In making the sibling correlations, values for age at attainment of specific stages of formation of individual teeth were converted into normalized *T*-scores, using McCall's method.[10,11] Since the *T*-scores thus obtained were both sex-specific and tooth-specific, male and female data could then be combined. Except for the use of *T*-scores, all statistical measures were of an elementary nature.[12]

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It should be noted that the maximum number of pairings in the present study follows the formula: $N \times N - 1/2$, when N is the number of siblings. Thus, for one family in the study, with 7 siblings, there are 21 possible sibling pairings. However, the theoretical maximum was not attained for any stage of the five mandibular teeth considered. In some cases $\overline{P}_2$ or $\overline{M}_3$ was congenitally absent. For some children the radiographs did not provide satisfactory data on age at attainment of particular stages of tooth development. Moreover, the very nature of the Fels Longitudinal Study, with its program of continuing recruitment, effectively limited the available data on the later stages of the later-forming teeth. The number of pairings used in each correlation are, therefore, given separately in the tabulations.

RESULTS

As is evident in Table 1, where sibling correlations are given for individual stages of tooth development, members of a sibship are consistently more alike than chance alone would allow. For 53 correlations where the number of sibling pairings exceeds 10 and are therefore detailed in Table 1, 23 correlations are significantly different from zero at the 5 per cent level of confidence. This may be compared with the 2 or 3 correlations that would be expected to achieve such levels if sibling resemblances were randomly distributed.

For the boys the sibling correlations average close to 0.30, with a mean correlation

TABLE 1

SIBLING SIMILARITIES IN TOOTH CALCIFICATION AND MOVEMENT

Mandibular Tooth and Stage	Boys		Girls		Mixed		All	
	No. Pairs	r	No. Pairs	r	No. Pairs	r	No. Pairs	r
P_1 beginning calcification	36	0.44*	12	0.46*	51	0.28*	99	0.33*
P_1 root formation	39	.30*	21	.24	56	.26*	116	.26*
P_1 apical closure	4	†	.....	.....	3	.....	7	.....
P_1 alveolar eruption	8	.....	6	.....	18	.17	32	.23
P_1 occlusal level	5	.....	3	.....	11	.39	19	.29
P_2 beginning calcification	38	.22	20	.32	61	.18	119	.19
P_2 root formation	29	− .09	20	.50*	50	.22	99	.18
P_2 apical closure	2	.....	1	.....	2	.....	5	.....
P_2 alveolar eruption	4	.....	4	.....	9	.....	17	.41*
P_2 occlusal level	4	.....	1	.....	5	.....	10	.46
M_1 beginning calcification	38	.....	11	.77*	46	− .003	95	.21*
M_1 root formation	42	.19	13	.77*	56	.45*	111	.40*
M_1 apical closure	14	.61*	2	.....	13	.44	29	.45*
M_1 alveolar eruption	11	.11	2	.....	13	.54*	26	.50*
M_1 occlusal level	10	.26	1	.....	7	.....	18	.35
M_2 beginning calcification	45	.09	20	.12	66	.18	131	.14
M_2 root formation	31	.31	21	.50*	49	.31*	101	.34*
M_2 apical closure	1	.....	1	.....	2	.....	4	.....
M_2 alveolar eruption	11	.69*	4	.....	14	.09	29	.50*
M_2 occlusal level	5	.....	.....	.....	5	.....	10	− .19
M_3 beginning calcification	22	−0.04	13	0.40	38	0.12	73	0.11
M_3 root formation	4	.....	1	.....	4	.....	9	.....
Mean r, all teeth	‡	0.29*	.....	0.51*	.....	0.24*	.....	0.28*

* Significant at $p = .05$.

† Correlations not reported where $N < 10$.

‡ Including all pairings.

coefficient (using all possible pairings) of 0.29. Female siblings tended toward higher values, with coefficients of similarity ranging from 0.2 to 0.77, and a mean r (for almost 180 separate pairings) of 0.51. With all possible pairings (brother-brother, sister-sister, and brother-sister) for all stages of tooth development and using all available data, the pooled mean coefficient of similarity was 0.28. This value, based on nearly 1,200 tooth-stage pairings, provides a good estimate of the degree to which siblings resemble each other in the timing of tooth development.

The data were separately analyzed, to determine whether similarities in tooth calcification (beginning calcification, root formation, and apical closure) were greater than

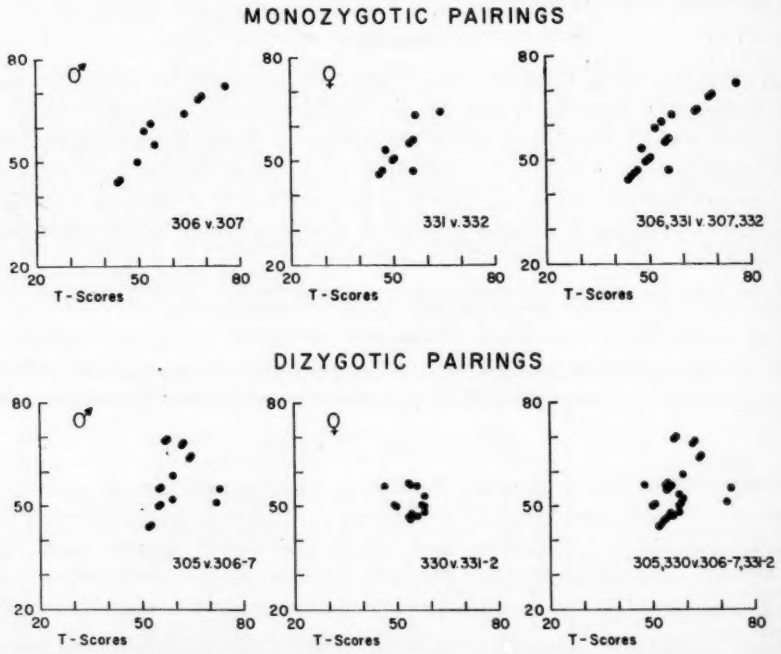

FIG. 1.—Comparative timing of tooth development in monozygotic pairings (*above*) and dizygotic pairings (*below*) from two sets of triplets comprising a monovular pair and a dizygote. Despite the common environment, the monozygotic pairs are far more alike in tooth development than are the dizygotic pairs from the same triplet sets. For the raw data, see Table 2.

those for tooth movement (alveolar eruption and attainment of the occlusal level). No evidence for this hypothesis was forthcoming. Similarly, the 53 correlations given in Table 1 were analyzed to determine whether later stages of tooth development exhibited greater degrees of sibling similarity. Again there was no evidence that, as children became older, they exhibited a higher degree of resemblance in the timing of tooth development.

Finally, sibling resemblances in two sets of triplets were analyzed. For one set of three boys (including a monozygotic pair and a dizygote) the correlation between the monozygotic pair was 0.95 (for 10 stages of tooth development), as contrasted with a correlation of 0.21 for 14 stages for the dizygotic pairings. For a similar monovular-

diovular female set of triplets, the monozygotic pairing exhibited a similarity coefficient of 0.79 for 10 stages of tooth development, as contrasted with —0.40 for 14 stages for the dizygotic pairings. Taken together, then, monozygotic pairings yielded a correlation of 0.91, as contrasted with 0.33 for the dizygotic pairings (see Fig. 1 and Table 2).

These triplet data further confirmed the hypothesis of genic determination for the timing of tooth development.

TABLE 2

COMPARATIVE DATA ON TOOTH FORMATION IN TWO SETS OF TRIPLETS

MANDIBULAR TOOTH AND STAGE	AGE AT ATTAINMENT IN YEARS					
	Triplets			Triplets		
	305*	306	307	330*	331	332
P_1 beginning calcification	.....	.....	.....	.....	2.5	2.5
P_1 root formation	7.5	7.0	7.0	7.0	7.5	7.5
P_1 alveolar eruption	14.5	.....	10.5	.....	.....	.....
P_1 occlusal level	15.5	.....	13.0	.....	.....	.....
P_1 apical closure	.....	12.5	12.5	.....	.....	.....
P_2 beginning calcification	.....	.....	.....	4.0	3.5	3.5
P_2 root formation	.....	.....	.....	8.0	8.0	8.0
M_1 root formation	4.5	4.5	4.5	4.5	4.0	4.0
M_1 alveolar eruption	.....	6.5	6.0	.....	7.0	7.0
M_1 occlusal level	.....	7.0	7.0	.....	8.0	7.0
M_1 apical closure	11.0	11.0	10.5	.....	.....	.....
M_2 beginning calcification	.....	6.5	7.5	4.0	3.5	3.5
M_2 root formation	9.5	11.0	11.0	9.5	9.0	8.5
M_2 alveolar eruption	13.5	14.0	14.0	.....	.....	.....
M_2 occlusal level	15.0	15.0	15.0	.....	.....	.....
M_3 beginning calcification	.....	.....	.....	10.0	9.0	10.0

* Dizygote.

DISCUSSION

As is evident in the preceding section, siblings are similar both in the timing of tooth calcification and in tooth movement. Taking all the data into consideration, sibling correlations prove to be of the order of 0.3, a value similar to that reported in many investigations of sibling similarities.

While this value of r is in accordance with a hypothesis of multigenic control (assuming little or no consanguinity), it is obviously necessary to rule out the role of common environment. Here several observations may be taken into account. First, sibling correlations in tooth calcification and tooth movement do not rise with increasing age for stage, as might be expected if communality of environment were an important factor. Second, other data on tooth formation suggest little or no influence of such variables as nutritional status or maturational level. For example, fatter children are not notably advanced in tooth formation, in our experience.

However, there are certain peculiarities of the data that merit attention. First, as is quite evident in the tabulations, female siblings are consistently more alike in the timing of tooth development. Second, monozygotic twins do not necessarily achieve perfect concordance in tooth formation, with over-all similarity coefficients of 0.79 and

0.95 for two sets of single-egg twins, as contrasted with similarity coefficients of 0.21 and —0.40 for dizygotic pairings from the same triplet sets.

The greater similarity of female sibling pairings is not in accordance with the hypothesis that tooth formation is exclusively influenced by autosomal genes and (like the sex difference in eruption and tooth size) tends to implicate the sex chromosomes. One distinct possibility is that the genotype XY introduces a greater degree of heterozygosity than does the XX genotype. Under these circumstances, it would be of considerable interst to know the rate of tooth development in Turner's syndrome* with the chromosomal type XO?, Klinefelter's syndrome† (of the type XXY), and in mosaics characterized by varying numbers of chromosomes.[13, 14, 15] Recent advances in cytogenetics may make it possible to answer such questions, and we have begun investigations along this line.

Since monozygotic twins are not completely "identical" in the rates of tooth formation and movement, these developmental phenomena cannot be exclusively gene-determined. However, the data strongly suggst that the rate of tooth development is largely, though not entirely, under genetic control, in the absence of endocrinopathy or chromosomal aberration.

Quite obviously, these findings must be considered as suggestive rather than conclusive. Despite the rather formidable number of sibling pairings, it is possible that another study might yield somewhat different results. Using other stages of tooth formation or motion or other samples, the correlation coefficients might be higher or lower. The hypothesis that the rate of tooth develpment in man is largely (but not entirely) gene-determined remains reasonable and is most likely correct.

SUMMARY

1. Sibling similarities in tooth calcification and tooth movement were investigated in 170 white Ohio-born siblings and two sets of triplets.

2. Sibling correlations for tooth calcification and tooth movement were positive, for the most part significantly different from zero, and of the order of 0.30.

3. Female pairings consistently exhibited higher sibling correlations in tooth development than did the male pairings.

4. Monozygotic twin pairings proved more alike in tooth formation ($r = 0.91$) than dizygotes ($r = 0.33$) from the same triplet sets.

5. Since body size, nutritional status, etc., have but little bearing on tooth development, these data suggest that the timing of tooth development is in large part gene-determined.

6. Analysis of tooth development in the genotypes XO, XXY, and in various mosaics was suggested as a further step in elucidating the problem.

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* Gonadal agenesis.

† Anatomical males lacking sperm-producing cells. In this condition there is a normal male XY chromosomal pair, plus an extra X.

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Experimental Radio-Osteonecrosis of the Jaws

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With the increased use of irradiation in the therapy of malignancies, an increasing number of cases of radio-osteonecrosis have appeared in the literature. The syndrome of radio-osteonecrosis has been known since 1927, when Baensch[1] reported the first case of fracture of the femoral neck following irradiation therapy for carcinoma of the cervix. There are now about a hundred reports of fractures of the neck of the femur following irradiation therapy and about sixty of radio-osteonecrosis of the mandible. Radio-osteonecrosis of the following bones has also been mentioned: maxilla, frontal, nasal, malar, mastoid, skull, rib, clavicle, metatarsal, pubis, ischium, ilium, and lumbrosacral spinous processes.

Despite the fact that the disease has been known for over thirty years, there is only one report[2] of an experimental investigation aimed at elucidating the pathogenesis of this disease. All the other articles have dealt with the clinical manifestations of the disease, and only a few of these articles have reported histopathological findings. A number of the publications cited in the present report[3–5] contain good reviews of the literature, and a complete discussion here would be redundant. A few pertinent facts may be drawn from the literature.

The relation between the dose administered and the occurrence of radio-osteonecrosis is not known. There have not been any reports, clinical or experimental, relating the amount of X-irradiation administered to the incidence of radio-osteonecrosis.

The time interval required for radio-osteonecrosis to become clinically manifested is variable and appears to depend upon the bone affected. Radio-osteonecrosis of the mandible is usually evident within 2 years following the irradiation, although there are a few cases of intervals as long as 13 and 15 years.[6] In the femur, radio-osteonecrosis is usually evident within 2–3 years following irradiation. The longest time interval is $9\frac{1}{2}$ years.[3] Radio-osteonecrosis of the rib and clavicle is usually evident within 4–5 years[7, 8] following irradiation, and 10–17 years[9] in the skull.

Radio-osteonecrosis has a variety of manifestations, and its clinical course is different in different bones. In the mandible it is a much more serious disease and a greater threat to life than it is with the other bones. Infections in the mouth, with all their sequelae, are constant occurrences, while infections in the femur, rib, clavicle, or calvarium are uncommon. Because of this complicating factor, mandibular destruction is usually extensive and requires radical treatment, like sequestectomy or mandibular resection. In addition, sequestra frequently produce fractures. The immediate cause

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of radio-osteonecrosis of the mandible in half the cases was tooth extraction, dental infection, denture trauma, or ligation of the external carotid artery.[6, 10] There is one report[5] of radio-osteonecrosis of the maxilla. In contrast with this, radio-osteonecrosis of the femur, clavicle, and rib becomes clinically manifested by spontaneous fracture.[3, 4, 7, 8] These fractures are not infected; they usually heal, but at a retarded rate; and fibrous union is not uncommon. According to Camp and Moreton,[9] lesions of the calvarium are asymptomatic and appear as multiple punched-out radio-lucencies discovered accidentally on routine examination.

The incidence of radio-osteonecrosis is relatively low and varies with the bones affected. In the femoral neck it varies from 0.9[11] to 7.5 per cent[12] and in the mandible from 5.6[10] to 24 per cent,[13] and in the calvarium it is reported to be about 0.25 per cent.[9] The incidence is based on relatively few reports and therefore cannot be construed as the true incidence among all the patients receiving external irradiation.

The histopathology of radio-osteonecrosis has been reported on the basis of biopsy, autopsy, or surgical resection; however, none of these specimens were taken from the jaws. In radio-osteonecrosis fractures, the histologic findings varied from "callus no different from that seen in non-radiated fractures"[14] to complete aseptic necrosis.[3] In all cases there was some degree of healing.

Based on observations in the femur, there are principally two concepts on the pathogenesis of postirradiation osteonecrosis. One is that the bone itself is the primary site of damage, and the other is that vascular obliteration is primary.

The present experiments were undertaken to elucidate (1) the relation between the quantity of fractionated irradiation and the deleterious bone changes known as "radio-osteonecrosis"; (2) the possible differences in the sensitivity of different bones regarding development of radio-osteonecrosis; and (3) the pathogenesis of the disease, particularly of the jaw. These points have been the object of this experiment.

EXPERIMENTAL METHODS

The experimental animal was the *Macacus rhesus* monkey. It was chosen for this work because it most closely resembles man in facial anatomy and dental development and therefore makes possible an experimental comparison with the clinical observations. Twenty monkeys were used, their ages varying from 15 to 18 months. Eighteen were irradiated, and two kept as controls. The monkeys had a deciduous dentition and about half had erupted maxillary and mandibular first permanent molars and incisors. The unerupted permanent teeth were in various stages of development, and the third molar was not yet evident.

Since this was the first experiment of its kind, the fractionated irradiation dose had to be arrived at empirically. It was decided to use the same quantity and quality of irradiation as that used in the hospital (University of Chicago Clinics) for the treatment of intra-oral malignancies. The physical factors of irradiation were: 250 KVP, 15 MA, H.V.L. 1.46 mm. Cu, F.S.D. 50 cm., dose rate 44.44 r/min, field of irradiation 3×4 cm. The field of 3×4 cm. involved the maxilla and the mandible mesiodistally from the canine tooth to the ascending ramus and superio-inferiorally from the zygoma to 1 cm. above the inferior border of the mandible. The center of the field was approximately at the occlusal level of the deciduous second molar. Irradiation from each side contributed to the total irradiation of the opposite side. The percentage contribution

from the opposite side was determined from a depth-dose chart that was calculated from a phantom model (Fig. 1). The animals were irradiated once a day, 5 days a week, alternating daily the right and left sides of the face.

As shown in Table 1, the animals were divided into three groups of 4,500, 5,500, and 7,500 r for the first irradiation. All animals demonstrated only epilation at the completion of the irradiation. On this basis they were thought to be more resistant to irradiation than had been anticipated. It was then decided to take three animals from each group and increase the irradiation, as shown for the second irradiation. The non-irradiation period indicated in Table 1 is the interval between the completion of the first irradiation and the beginning of the second.

Diagnostic X-rays of the lateral-jaw type were taken at regular intervals to observe the effects of infection, sequestrum formation, regeneration, tooth development, and tooth eruption. At the completion of the experiments the animals were killed with an overdose of Nembutal. Tissues were fixed in 10 per cent neutral formalin. The jaws

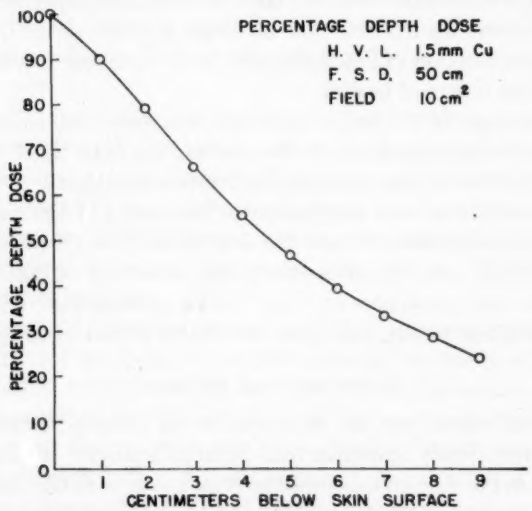

FIG. 1.—Depth-dose chart calculated from a phantom model

TABLE 1

IRRADIATION SCHEDULE

No. Animals	First Irradiation			Second Irradiation		
	r/Day	Dose to Bone (r)	Non-Irradiation Period	r/Day	Dose to Bone (r)	Total Dose to Bone (r)
3.....	207	4,500	.....	.....	.....	4,500
3.....	218	5,500	.....	.....	.....	5,500
3.....	224	7,500	.....	.....	.....	7,500
3.....	214	4,500	60	300	4,000	8,500
3.....	218	5,500	46	300	4,000	9,500
3.....	224	7,500	30	300	3,500	11,000

were cut into small pieces. The bone was decalcified in formic acid–sodium formate solution. Tissues were embedded in celloidin, cut at 8–12 μ, and stained with hematoxylin eosin–azure II.

GROSS OBSERVATIONS

Figure 2 is a graphic summary of the gross observations of the effects of fractionated irradiation on all the experimental animals. The progress of the lesions, time of tooth extraction, roentgenographic evidence of sequestrum formation, and time of killing of the animals are plotted against the irradiation dosage.

About 38 days after the beginning of the irradiation, epilation was observed in the irradiated area after 4,300–4,500 r had been delivered. No other changes were evident after the completion of the first irradiation of all six groups. The irradiation of the 4,500–9,500 r groups was completed without any outward changes in the tissues. In the 11,000 r group, one monkey had necrosis of the cheek (Fig. 3), and the other two had necrosis of the gingiva when this irradiation dose level was reached. Diagnostic X-rays showed no changes in the hard tissues of any of the animals at this time.

The time of onset of necrosis and the dosage of irradiation were in direct relationship to each other—namely, necrosis in the 11,000 r group began the day that irradiation

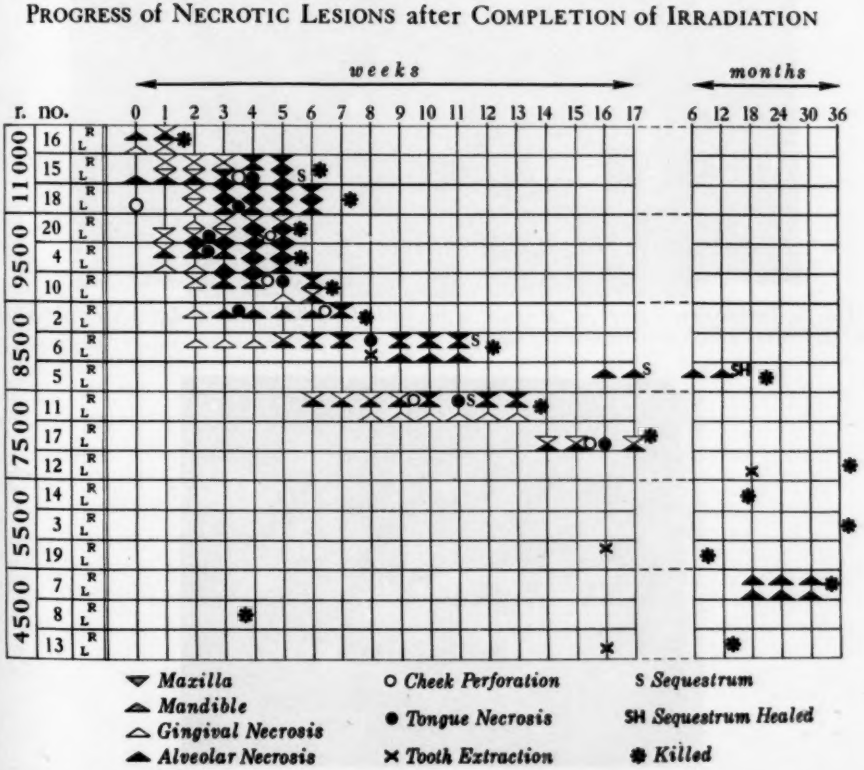

FIG. 2.—Chart of onset, progress, and severity of gross necrotic lesions in relationship to time and irradiation dosage in the *Macacus rhesus* following external irradiation.

was completed, 1 week later in the 9,500 r groups, 2 weeks later in the 8,500 r group, and 6 and 14 weeks later in the 7,500 r group (Fig. 2). There was no necrosis in the 5,500 r group. In the 4,500 r group, one animal had necrosis beginning 1 year and 7 months after irradiation. The severity and extent of the necrosis were also in direct relationship to the time of onset, which was related to the irradiation dose. In the animals irradiated with the two highest levels—9,500 and 11,000 r—the necrosis had a very rapid course. It became bilateral early, usually involved the maxilla and tongue, and produced a perforation of the cheek. The animals did not survive long enough to form a sequestrum, but incipient sequestrum formation, as evidenced by a break in the inferior border of the mandible, was noted in two of the animals. All these animals

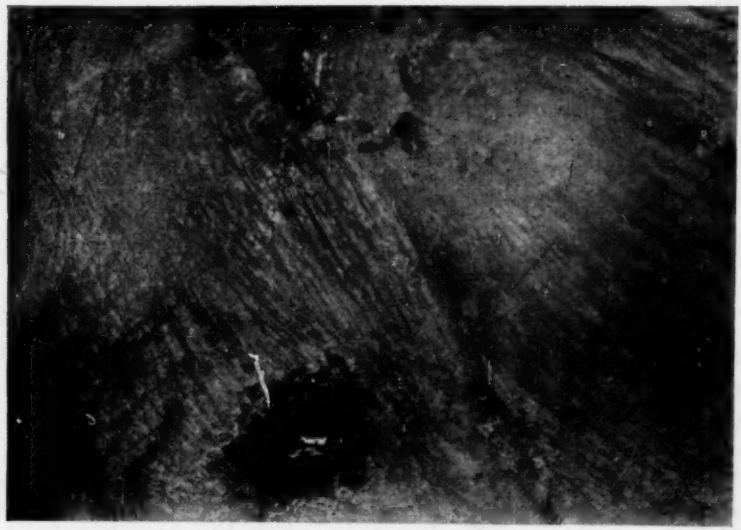

FIG. 3.—Perforation of cheek in the center of the field of irradiation. Three days after the first sign of necrosis and 3 days postirradiation. 11,000 r.

FIG. 4.—Early sequestrum, with mandibular fracture in bicuspid area and resorption of cortical plate in ascending ramus. Four weeks postgingival necrosis and 5 weeks postirradiation. 9,500 r, post mortem.

were killed within 7 weeks of the completion of irradiation. In the middle-range dose levels—7,500 and 8,500 r—the necrosis proceeded at a slower rate, was usually unilateral, and involved the maxilla, tongue, and cheek of only half the animals. The more indolent course of the necrosis in this dose range permitted time for sequestrum formation. Three of the five animals with necrosis developed sequestra involving half the mandible (Figs. 4–6). A sequestrum of this size corresponds roughly to the size of the field of irradiation. Necrosis in the lowest dose range was found in only one animal, and it followed a slow course that was confined to the mandibular alveolar ridge.

The necrotic lesions in all cases showed a similar pattern. The lesion always began as a unilateral mandibular necrosis. The first changes occurred in the interdental papilla of the lower second deciduous molar and the first permanent molar (Fig. 7). From this point the necrosis extended to involve the cervical gingiva, the attached gingiva, and the buccal mucosa (Fig. 8). Concurrently, the lingual aspect of the mandible became involved, but at a slower rate and at a later time than the buccal aspect. As the necrosis progressed, it extended more posteriorly than anteriorly. The

FIG. 5.—Advanced sequestrum formation. Note fracture in sigmoid notch. Eight weeks postgingival necrosis and 14 weeks postirradiation. 7,500 r, post mortem.

FIG. 6.—Advanced sequestrum formation. Area corresponds to field of irradiation. Ten weeks postgingival necrosis and 12 weeks postirradiation. 8,500 r, post mortem.

FIG. 7.—Early gingival necrosis with exposure of alveolar crest. One and one-half weeks after onset of interdental papilla necrosis and postirradiation. 11,000 r, post mortem.

FIG. 8.—Advanced gingival and bone necrosis. The lesion does not extend anterior to the field of irradiation, and the maxilla is only minimally involved. Same animal as in Fig. 7 but opposite side of mouth.

necrotic lesions never extended anterior to the field of irradiation, that is, anterior to the canine tooth. The retromolar area and ascending ramus were always involved in those cases where there was extensive necrosis (Fig. 9). In the maxilla the necrosis was usually confined to the interdental papilla and cervical gingiva. The palate was involved only in cases of extensive necrosis. In the beginning, the necrosis progressed slowly, although steadily; secondary infection superimposed on the necrotic lesion markedly accelerated the process; and extensive cellulitis sometimes occurred, with perforation of the cheek and early sequestration of the bone. The degree of cellulitis was usually proportional to the time at which perforation and sequestration occurred. All animals which developed a sequestrum were killed before exfoliation of the sequestrum because of physical debilitation, except one which survived. This animal received

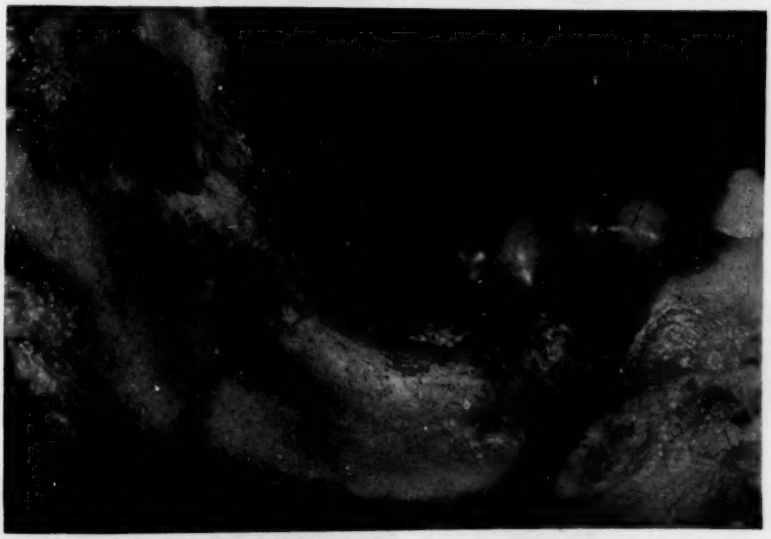

FIG. 9.—Extensive necrosis of mandible. The necrosis did not extend anterior to the field of irradiation. Five weeks after onset of gingival necrosis and 6 weeks post-irradiation. 9,500 r, post mortem.

8,500 r and developed a unilateral necrotic lesion in the gingiva 14 weeks after irradiation. One week later there was roentgenographic evidence of sequestration. Six months elapsed before the sequestrum was sufficiently isolated to be removed. Bone regeneration at the inferior border could be observed 1½ months after sequestration was evident and continued after the sequestrum was removed. One year after the roentgenographic evidence of sequestration, the mandible showed almost complete regeneration in height and width (Figs. 10–15). The soft tissue healed simultaneously with the bone.

It is a common experience that tooth extraction frequently results in the manifestation of the osteonecrotic process in individuals previously subjected to X-ray treatment in the jaw region. It was thought desirable to determine whether tooth extraction in this experiment would produce similar results. It is of particular interest to determine at which time a dormant osteonecrosis can be evoked by this treatment and to detect in this way early lesions which would otherwise remain obscure. Four extractions

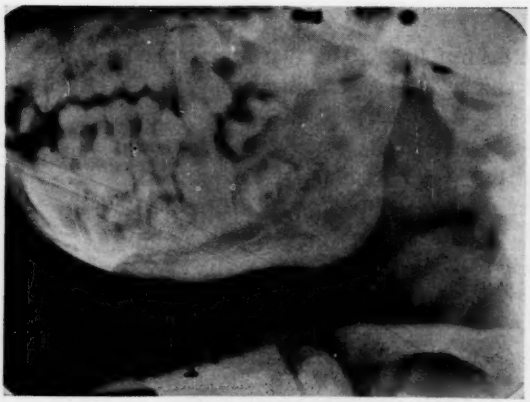

Fig. 10

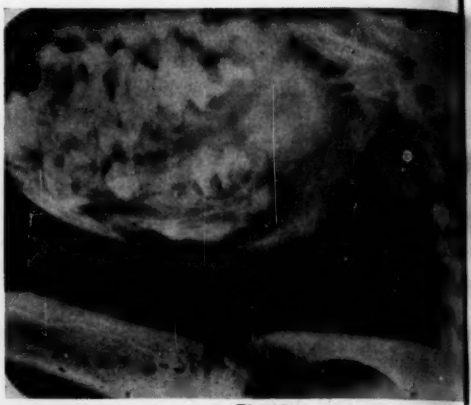

Fig. 11

Fig. 12

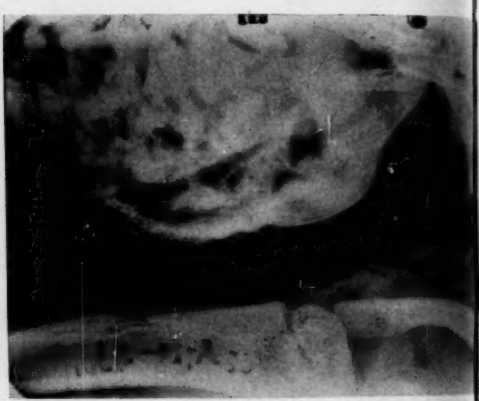

Fig. 13

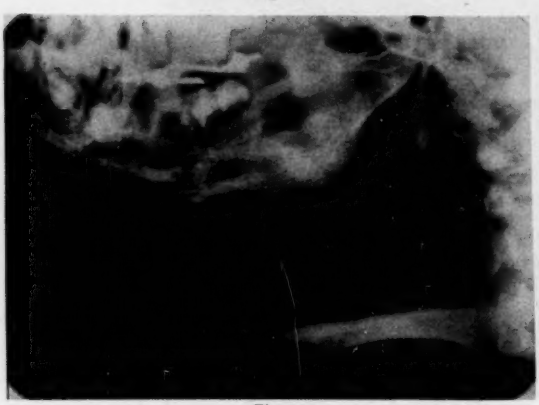

Fig. 14

Fig. 15

FIGS. 10–15.—Roentgenographs of a monkey that received 8,500 r and developed radio-osteonecrosis of the right m dible. FIG. 10: Normal mandible. Three months postirradiation. FIG. 11: Sequestrum formation with fracture of the ferior border of the mandible. One week postgingival necrosis and 4 months postirradiation. FIG. 12: Regeneration of inferior border, with near closure of the fractured ends. Three months postgingival necrosis and 7 months postirradia FIG. 13: Fractured inferior border healed with callus formation. Sequestrum in process of exfoliation. Five months p gingival necrosis and 9 months postirradiation. FIG. 14: Mandibular repair completed, and sequestrum exfoliated. S months postgingival necrosis and 11 months postirradiation. FIG. 15: Repaired mandible, 16 months postgingival nec and 20 months postirradiation. Post mortem.

of first molars were performed: one in the 4,500 r group, one in the 5,500 r group, one in the 7,500 r group, and one in the 8,500 r group (Fig. 2). The animals in the first three groups did not have any gross or roentgenographic evidence of osteonecrosis at the time of extractions. Extractions were done 4 months after irradiation in the 4,500 and 5,500 r groups and 19 months after irradiation in the 7,500 r monkey. The wounds healed normally, and no changes developed subsequently (Fig. 16). The animal in the 8,500 r group showed no visible lesions at the site chosen for the extraction, but there was gingival necrosis at the opposite side, with exposure of bone but without bone changes recognized by X-ray. The lower left first permanent molar at the normal-appearing site was extracted 2 months after irradiation. The extraction wound did not heal, and necrosis of the gingiva and sequestration of the tooth socket developed in the jaw about 20 days after extraction (Fig. 17).

In all cases the bone changes occurred only after gingival lesions had developed, and no bone changes were found roentgenographically under an intact gingiva. The soft-tissue necrosis began invariably in the interdental papillae and extended to involve the

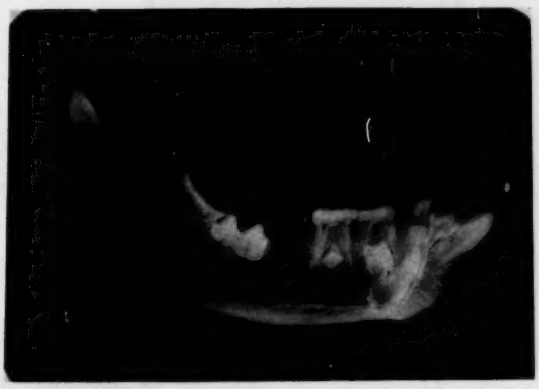

FIG. 16.—Healed extraction wound and irradiation-produced cessation of further tooth formation. Ten months postextraction and 14 months postirradiation. 4,500 r, post mortem.

FIG. 17.—Sequestrum of tooth socket following extraction of first permanent molar. Four weeks postextraction and twelve weeks post irradiation. 8,500 r, post mortem.

gingival sulcus, adjoining soft tissues, and underlying bone. The dominant feature of the clinical and roentgenographic changes observed in the soft tissues as well as in the bone was an infectious process. It appears that the interdental papilla and the gingival sulcus constitute *Loci minoris resistentiae* from which a normally present bacterial flora could spread and cause a secondary infectious process involving the bone.

The irradiation completely inhibited further tooth development and destroyed the anlage of the third molar tooth. The size and shape of the permanent teeth were therefore dependent upon their state of development at the time of irradiation. Most of these partially formed teeth erupted into occlusion and were firmly held by the gingiva. The details and significance of this observation on tooth formation and eruption will be reported separately.

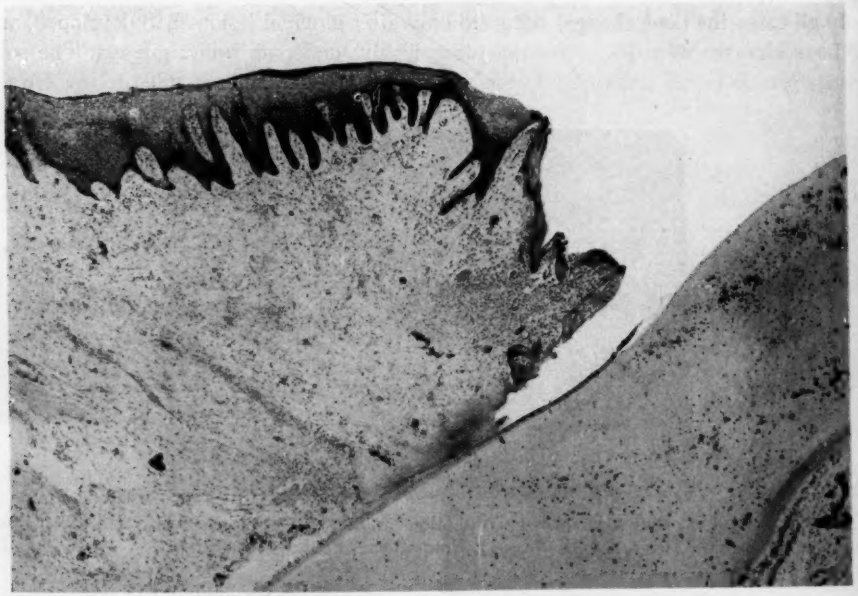

FIG. 18.—Early gingival necrosis. Six weeks postirradiation. 9,500 r. ×65

HISTOLOGIC OBSERVATIONS

Because of the destructiveness of the necrotic lesions at the high dose levels, it is generally not possible to relate precisely the histologic sequence of events with the irradiation dose and time sequence. The progress of the changes can often be determined better on the basis of the composite histologic observations than by the chronologic order of the material.

The interdental papilla was the first area in which inflammation and necrosis were observed. The first changes seen were an increase of lymphocytes and dilatation of blood vessels in the corium. The epithelial attachment proliferated downward along the root surface and in so doing became thin and infiltrated with lymphocytes. Next there were ulceration of the epithelial attachment and necrosis of the underlying connective tissue (Figs. 18 and 19). The inflammation, with subsequent necrosis, might then

spread by two routes: via the periodontal ligament or via the gingival tissue, the most common being by way of the periodontal ligament. Via this route the periodontal ligament showed the greatest destruction, and the contiguous structures were involved by direct extension. When gingival tissue was the primary route of spread, the necrosis extended in a linear fashion lateral to the periosteum in an inferior, anterior, and posterior direction (Fig. 20). The necrosis spread rather widely under an intact epithelium, and the contiguous structures were involved by direct extension. In these cases the periosteum and bone were usually involved by the necrotic process before the involvement of the epithelium. Regardless of the route of spread, the necrosis was always in advance of the grossly observed lesion. In the bone the necrosis frequently

FIG. 19.—Early gingival necrosis slightly more advanced than Fig. 18. Six weeks postirradiation. 9,500 r. ×65.

spread via the marrow spaces (Fig. 21) and occasionally via the Haversian canals. While the necrosis involved the ascending ramus in several of the animals, it never involved the region of the incisors, and in only a few cases did it involve the canine area. In these few cases the necrosis extended into the canine area via the marrow spaces.

Whether the inflammation and necrosis spread via the periodontal ligament or gingival tissue, the reaction of the bone was the same: the osteocytes adjoining the necrotic process were usually pyknotic but sometimes normal-appearing; osteocytes two, three, or more lacunar spaces removed from the necrotic process were usually normal-appearing; marrow spaces adjoining the necrosis were filled with chronic inflammatory cells, mostly lymphocytes. Concomitantly, osteoclastic bone resorption was taking place at the immediate periphery of the necrotic lesion via marrow spaces and Haversian canals.

As expected, histologic evidence preceded roentgenographic evidence of sequestration (Fig. 22). This was clearly shown in two animals which demonstrated roentgenographi-

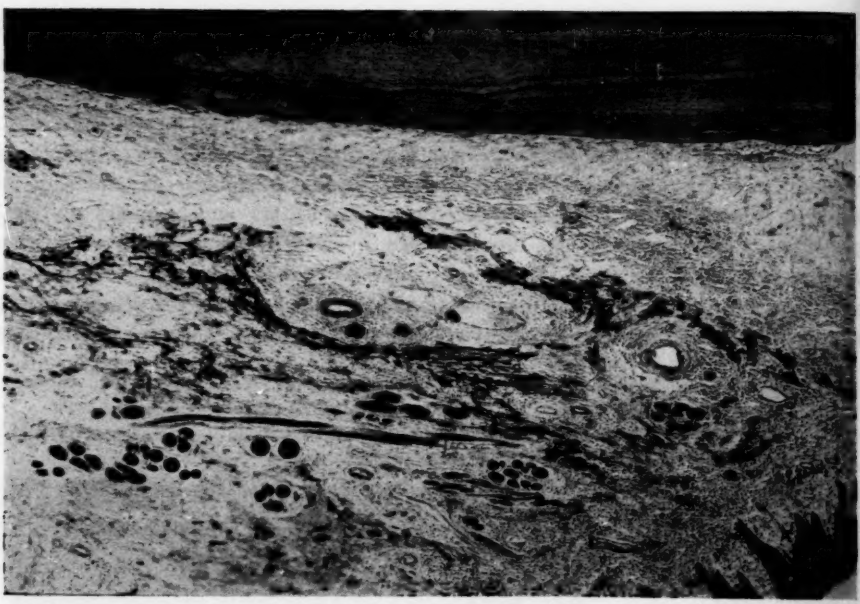

FIG. 20.—Advanced soft-tissue necrosis not involving periosteum. Six weeks postirradiation. 11,000 r. ×65.

FIG. 21.—Extension of necrosis via marrow spaces, with normal intact gingiva. Six weeks postirradiation. 9,500 r. ×38.

cally a break in the inferior border of the mandible in the area of the second biscuspid, but the remainder of the mandible appeared normal. Microscopic examination of these mandibles revealed sequestrum formation extending from the bicuspid area to the ascending ramus and involving the inferior border. There was periosteal bone formation at the periphery of the sequestrum. Another animal with extensive necrosis had a roentgenographically normal mandible, but histologically there was sequestrum formation extending from the bicuspid area to the ascending ramus.

Irradiation produced vascular changes that were found predominantly in the mandible and in the field of irradiation. The changes involved primarily the arterioles, while the medium- and large-sized arteries were only occasionally involved (Fig. 23), and the

FIG. 22.—Resorption of bone in Haversian canals at the inferior border of the mandible at the periphery of the field of irradiation. One and one-half weeks postirradiation. 11,000 r. ×100.

capillaries and veins were not affected. The intima and media of the vessel wall increased in size, with a proportional decrease in size of the lumen (Fig. 24 and 25). This thickening of the arteriolar walls varied from slight to complete occlusion and resembled arteriolarsclerosis. The medial and intimal proliferations were found early and progressed rapidly. They were seen as early as 1½ weeks, and vascular occlusion occurred within 5–7 weeks postirradiation. Inflammation and necrosis complicated the interpretation of the vascular changes. However, the vascular changes were found bilaterally in cases of unilateral necrosis and also in early necrotic lesions where the necrosis was confined to the tooth. Consequently, it must be assumed that the vascular changes were primary irradiation effects.

Histologically, a sterile necrosis was found under an intact gingiva in two animals which had received 7,500 r. The necrosis was confined to the inferior third of the

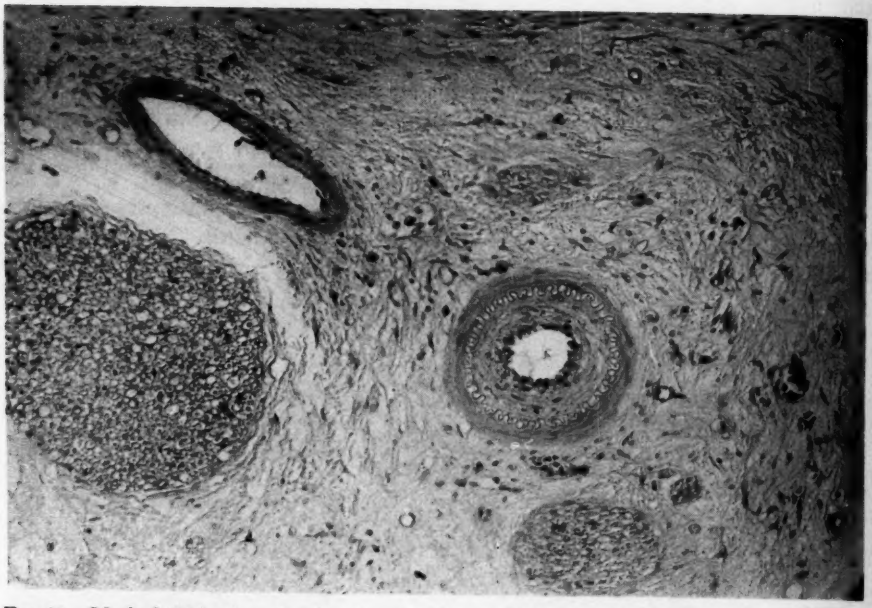

FIG. 23.—Marked thickening of main artery in the mandibular canal. Normal vein and capillaries. Eight weeks postirradiation. 8,500 r. ×200.

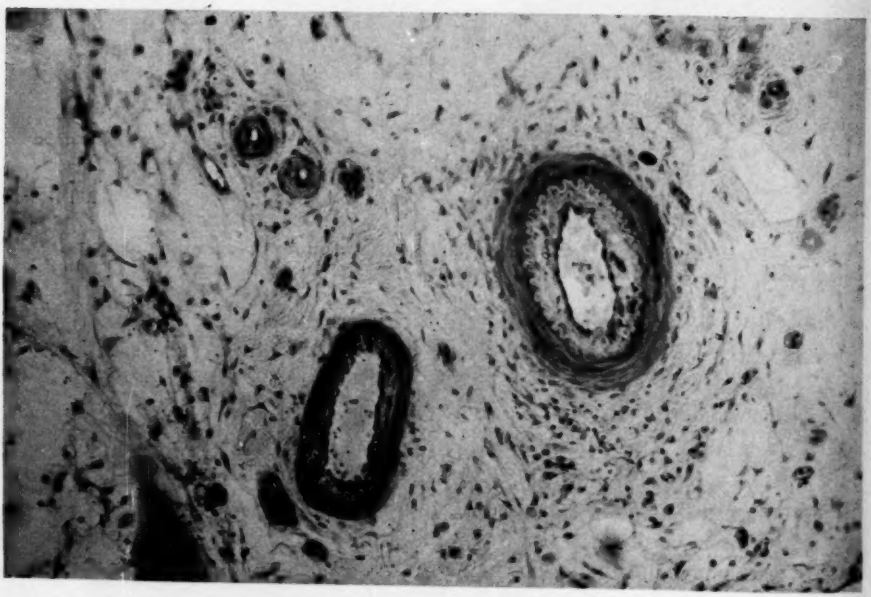

FIG. 24.—Arterio- and arteriolarsclerosis. Six weeks postirradiation. 11,000 r. ×200

mandible in the molar area and was most prominent in the cortical plate of the mandibular border (Fig. 26). The periosteum was normal. The middle and superior parts of the mandible had normal-appearing osteocytes and Haversian canals, and there was new bone formed in the middle of the mandible. The arterioles in these areas had markedly thickened medial and intimal layers. There was no evidence of aspetic necrosis in the maxilla.

The destructive process in the alveolar portion of the mandible produced an exclusively periosteal reaction at the inferior border of the mandible. New trabeculae of bone were laid down upon the cortical plate of the inferior border (Fig. 27), the osteocytes of which appeared vital in most of the animals. The amount of new bone

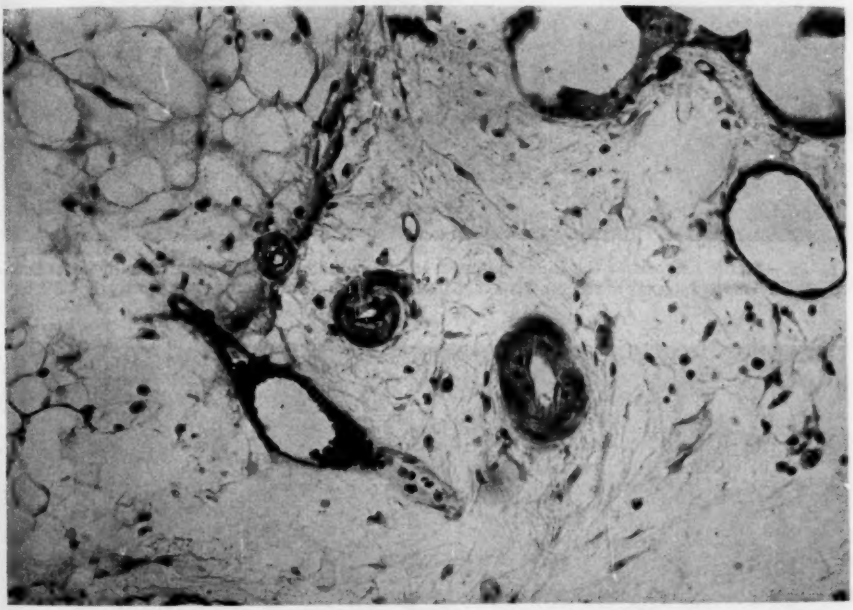

FIG. 25.—Arteriolarsclerosis with normal capillaries. Seven weeks postirradiation. 11,000 r. ×300

formation was directly proportional to the destruction of bone by the necrotic process and to the chronicity of the lesion. If the infection was quickly overwhelming, little or no bone was formed, but the longer the time from the onset of the infection, the greater the bone formation. However, if the time was the same, the formation of bone would be greater at the site of more intensive inflammation and necrosis. This periosteal bone formation was responsible for the complete regeneration of the almost entirely sequested mandible in one monkey (No. *5* in Fig. *2*). Microscopically, the mandible was composed of normal cortical and cancellous bone (Fig. 28). Most of the arteries and arterioles in the body of the mandible had markedly thickened walls, and the adventitia was greatly increased in both density and size. In the ascending ramus much of the marrow had been replaced by bone, giving it an eburnated appearance. The left mandible did not have any clinically evident necrosis, and histologically it had changes that were different only in degree from the right mandible. Some of the arteri-

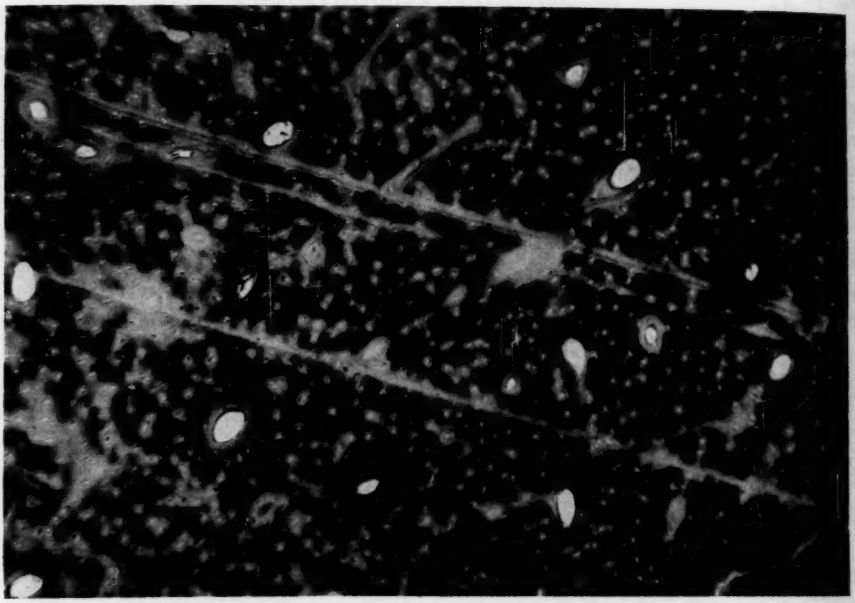

FIG. 26.—Sterile necrosis at the inferior border of the mandible. Intact gingiva with no inflammation. Seventeen weeks postirradiation. 7,500 r. ×200.

FIG. 27.—Marked amount of bone formation at the inferior border of the mandible, an exclusively periosteal reaction. Four weeks postnecrosis, 6 weeks postirradiation. 9,500 r. ×27.

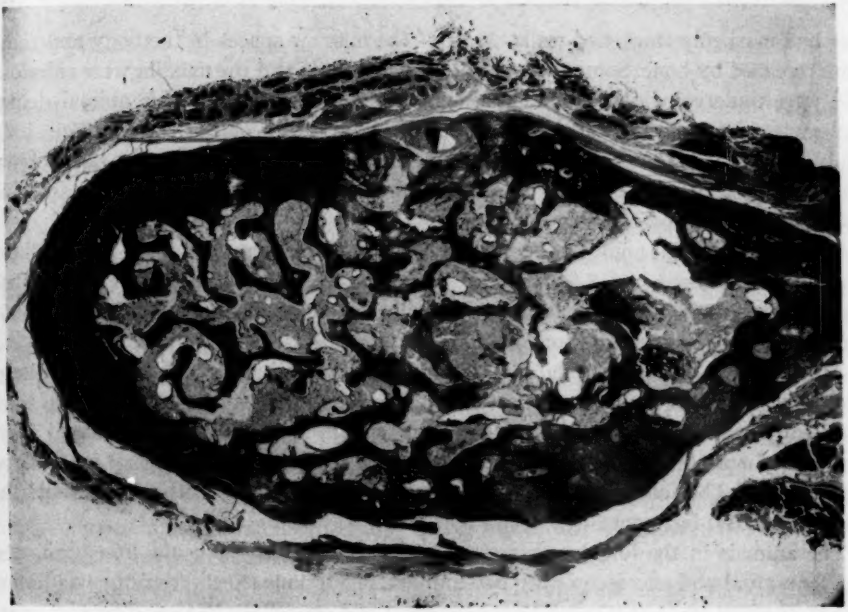

FIG. 28.—Repaired mandible. Most of the marrow is gelatinous, many dilated sinuses evident. Twenty months postirradiation. 8,500 r. ×14.

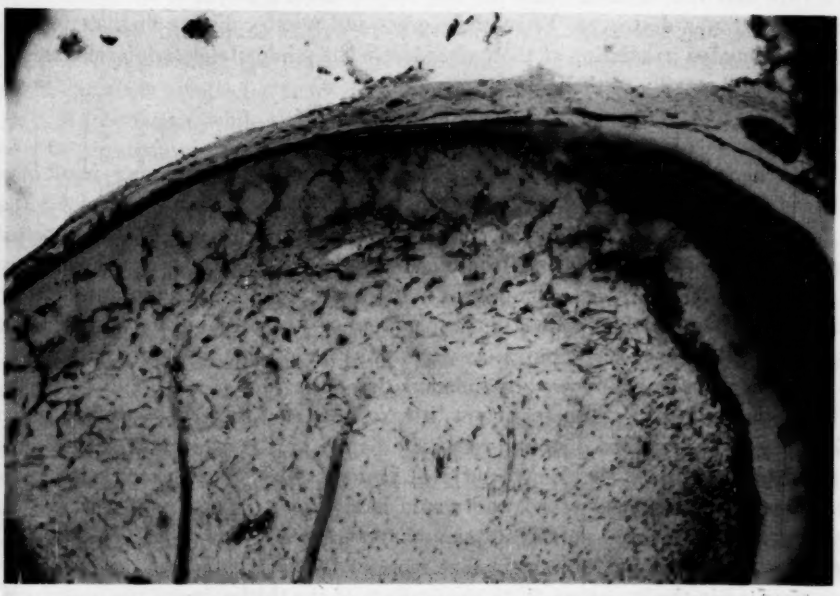

FIG. 29.—Irradiation-produced osteodentin. Odontoblasts end abruptly at the junction of normal and abnormal dentin. Five and one-half weeks postirradiation. 9,500 r. ×110.

oles had markedly thickened walls. Most of the marrow spaces in the body and ramus were replaced by bone. Some teeth in both the mandible and the maxilla were ankylosed and were undergoing resorption, with many osteoclasts present. This animal demonstrates two important points: (1) if the infective nature of the osteonecrotic lesion could be arrested, the mandible contained sufficient regenerative powers to overcome the deleterious effect; (2) the marked thickening of some arteries and arterioles was not sufficient to produce an ischemic necrosis.

The marrow in all animals which received 7,500 r or more was fatty and acellular, except one which received 8,500 r and had a regenerated mandible. In this animal there was hematopoiesis in the mandible and maxilla. The animals in the 5,500 and 4,500 r range had hematopoiesis in both mandible and maxilla.

The dental structures exhibited characteristic irradiation effects. The altered odontoblasts were associated with osteodentin formation (Fig. 29), and the pulp tissue in the area was of a more fibrous nature. The epithelial root sheath terminated at the junction of normal dentin and osteodentin, which resulted in cessation of further root development. The follicular sac was attached to the cementum without differentiating into periodontal ligaments.

The animals in the low-dose range of 4,500 and 5,500 r, with one exception, were grossly normal and microscopically had minimal deleterious effects from the irradiation. The epithelium was intact, with normal cortical and cancellous bone and hematopoietic marrow. There was no bone formation at the inferior border. Only a few arterioles had a moderately thickened wall, and all the other blood vessels were normal. The only structure that was strikingly affected was the developing tooth, in which further root development was destroyed. From these gross and microscopic findings, it is obvious that fractionated irradiation at these dose levels has minimal deleterious effects on the mandible and maxilla of the monkey.

DISCUSSION

From the foregoing observations it is clear that changes were occurring simultaneously in several tissues during and after irradiation. In the high dose levels of 9,500 and 11,000 r the pathologic changes occurred early, and the osteocytes demonstrated a greater degree of change than did the blood vessels. Also, in these dose levels a rampant infectious process was prominent. In the lower dose levels and consequently at long survival periods, there were still many pyknotic osteocytes, but the vascular lesions were more prominent. The gingiva, as well as the periosteum, appeared normal at all dose levels except when involved by inflammation or necrosis.

From the findings in this experiment, it may be stated that radio-osteonecrosis resulted primarily from the direct effect of irradiation on the osteocytes. The osteocyte damage persisted into the longer survival periods, when vascular changes became prominent. The thickened vessel wall would likely result in decreased vascular flow, and, therefore, decreased nutritional supply to the area seems probable. It is at this chronic stage of radio-osteonecrosis that most of the histopathologic specimens have been reported in the literature. With the findings of the thickening of the vascular wall and pyknotic or absent osteocytes, it is easy to understand why vascular changes were thought to be primary in the production of radio-osteonecrosis. The present experiments demonstrate that vascular damage is not primary. Vascular damage does, how-

ever, appear to be important in the continuance of the deleterious changes in the osteocytes.

Damage to the gingiva is not necessary to the production of radio-osteonecrosis, as is demonstrated in two experimental animals with sterile osteonecrosis under an intact gingiva. However, disruption of the gingival surface made a dormant radio-osteonecrosis grossly evident. This rupture of the gingival surface usually occurred spontaneously at the gingival sulcus, probably because it received a higher quantity of irradiation due to the secondary rays arising from the enamel. Tooth extraction will also rupture the gingival surface and therefore result in clinically evident radio-osteonecrosis. The disruption of the gingival surface appears to be the only function of tooth extraction in the production of radio-osteonecrosis. Trauma from artificial dentures will likewise result in radio-osteonecrosis by causing a break in the gingival surface. This has been reported in the literature,[6] and the author has had similar clinical experience.

The observation that the mandible was more frequently and more extensively involved by radio-osteonecrosis than the maxilla suggests the possibility that these two bones respond differently to irradiation. As mentioned previously, maxillary necrosis always followed mandibular necrosis and had a more restricted and indolent course. The maxilla showed less damage histologically from irradiation.

It seems unlikely that there would be an essential difference in the radiosensitivity of the osteocytes of the mandibular bone as compared with the osteocytes in maxillary bone. The anatomic structure of the bone may be a factor in irradiation difference between mandible and maxilla. The mandible consists of much more compact bone than the maxilla, and, because of the scattering of irradiation, the osteocytes of the mandible may receive more irradiation. The effect of irradiation on the blood supply must also be considered in assessing the different susceptibilities of mandible and maxilla. The mandible has a more restricted or localized blood supply, which is almost completely in the field of irradiation, while the maxilla has a much more diffuse blood supply, with many more anastomoses located outside the area of irradiation. This may be the reason that the mandible suffered greater vascular and therefore greater nutritional damage, as well as greater direct irradiation damage to the osteocyte.

In considering biological reasons for the difference in irradiation effects on the mandible and maxilla, it must be noted that the irradiation traversed more bone in the maxilla than in the mandible and, consequently, that the exit dose of the maxilla was probably less than that of the mandible. The same portals were used for the entrance and exit doses of the mandible and maxilla, and the percentage transmission of irradiation was based on the depth doses calculated from a phantom model (Fig. 1).

The actual difference between the exit dose of the maxilla and that of the mandible could not be measured with a sufficient degree of accuracy. It is therefore possible that the difference between mandible and maxilla was due more to the difference in irradiation, than to biological, factors.

Fractionated irradiation of 7,500 r or more under these experimental conditions is required for the production of radio-osteonecrosis of the mandible and maxilla in the *M. rhesus* monkey. Only Wildermuth and Cantril[5] report tissue doses to the mandible, and they ranged from 4,100 to 6,300 r. In the present experiments, one animal out of six in the low-range group (5,500 and 4,500 r) developed radio-osteonecrosis. This is roughly in the same order of magnitude as Wildermuth and Cantril's report of seven

cases of radio-osteonecrosis out of one hundred and four patients. Most of the lesions in these experiments occurred earlier and ran a much faster course than did the lesions reported in man. Some of the lesions in the middle-range group (7,500 r) followed a course that was comparable to clinical reports in man. The irradiation doses of 11,000 and 9,500 r produced a massive destruction of all tissues at the site of irradiation that was already evident at the time of completion of irradiation or shortly thereafter.

SUMMARY

The maxilla and mandible of eighteen *M. rhesus* monkeys were subjected to external fractionated irradiation ranging from 4,500 to 11,000 r.

The time of onset of necrosis and the dosage of irradiation were in direct relationship to each other. The severity and extent of the necrosis were in direct relationship to the time of onset.

The gross necrotic lesion always began in the interdental papilla of the mandibular molar that was in the center of the field of irradiation. From this point the necrosis extended to involve the cervical gingiva, the attached gingiva, and the buccal mucosa. In the maxilla the necrosis was confined to the interdental papilla and cervical gingiva. The palate was involved only in case of extensive necrosis.

Microscopically, the interdental papilla was the first area that demonstrated inflammation. This condition was followed by ulceration of the epithelial attachment and necrosis of the underlying corium. The necrosis spread by two routes: via the periodontal ligament and via the gingival tissue. The most common route of extension was via the periodontal ligament, with the contiguous structures involved by direct extension.

Radio-osteonecrosis appears to result primarily from the direct effect of irradiation on the osteocyte. Changes in the blood vessels played a role in the aggravation and prolongation of the irradiation effects. Radiation produced marked thickening of the walls of arteries and arterioles. The veins and capillaries were not affected. There was no evidence of ischemic necrosis.

Radio-osteonecrosis can occur under an intact gingiva. A dormant or latent radio-osteonecrosis requires disruption of the gingival surface to become grossly evident.

With a sufficiently long period of time, a restoration of the radio-osteonecrotic damage is possible.

The difference between the irradiation effects on the mandible and maxilla is discussed.

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Studies on Rehardening of Artificially Softened Enamel

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A method has been found by which artificially softened enamel can be rehardened in solutions of dicalcium phosphate dihydrate adjusted to pH 6.8–7.3.

About one hundred human teeth from the Birmingham area were collected and stored in tap water with thymol crystals. Sound teeth were ground and polished to provide flat surfaces, and their hardness was determined by using the Kentron Micro-Hardness Tester, as described by Newbrun, Timberlake, and Pigman (*J. D. Res.*, **38**:295, 1959). The hardness was measured initially, after softening, and after a rehardening treatment. The softened teeth were prepared in groups of four

REHARDENING OF ARTIFICIALLY SOFTENED ENAMEL

Rehardening Solution		Average Hardness (K.N.±S.D.)			Average Hardness Recovery (Per Cent)
$CaHPO_4 \cdot 2H_2O$ (mM/l)	pH	Initial	After Softening	After Rehardening	
1.5	7.3	309±8	199±10	258±17	54
1.5	7.3	291±9	187±10	265±14	75
2.0	7.0	320±7	228±13	328±48	109
2.0	7.0	316±10	234±9	322±29	107
2.0	7.2	276±8	163±9	252±24	79
2.0	7.2	302±10	203±6	245±21	42
2.5	6.8	275±6	190±13	299±19	128
2.5	6.8	316±9	168±4	282±22	77
3.0	6.8	306±10	223±8	322±27	119
3.0	6.8	300±5	190±8	298±17	99

to eight by treatment with stirred acetate buffer, 0.001 M at pH 5.50 for 2–6 hours at 37° C.

After considerable preliminary work, rehardening could be accomplished under the following conditions (see table). Solutions of dicalcium phosphate dihydrate at the concentrations given in the table were prepared by dissolving the salt in twice this molar concentration of HCl and raising the pH by addition of KOH (0.05 N.). Softened teeth in groups of two to four were placed in 500 ml. of the freshly made rehardening solution, which was replaced each day and maintained at 37° C.

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The Effect of Irradiated Yeast on Salivary Glands and Dental Caries in the Rat

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In a previous report (*J. D. Res.*, **38**:1052, 1959) the addition of 10 per cent irradiated yeast to a stock corn diet significantly increased its cariogenic effect as well as significantly inhibiting the growth of rats. When ashed yeast was used, however, both the cariogenic and the growth-inhibiting effects were lost, thus suggesting that a heat-labile factor is responsible for these biological effects. Since the mechanism responsible for producing these effects is not known, it was considered important to learn the effect of yeast on the thyroid and salivary glands.

Forty male weanling Sprague-Dawley strain rats were divided into two groups according to initial body weight. Group I received a cariogenic stock corn diet. Group II received the same diet except that sufficient irradiated yeast was added at the expense of corn to increase the level to 20 per cent. Both groups received the same low-fluoride drinking water and their respective diets ad libitum. All

Group	No. of Rats	Mean Final Body Weight (Gm.)	Mean No. Lesions	P*	Severity† Index
Control.....	14	282	7.4±1.0‡	.....	1.85
20% yeast....	14	191	9.7±1.1	0.04	2.51

* Calculated on the basis of the Student "T" test.

† See *J.A.D.A.*, **46**: 290, 1953.

‡ Standard deviation.

the animals were housed in raised screen cages in an air-conditioned room. After 100 experimental days the animals were sacrificed, the heads removed for dental caries evaluation (*J. D. Res.*, **33**:33, 1954), and the thyroid and salivary glands fixed for histologic examination.

The data obtained are seen in the table. As reported previously, the animals which received the irradiated yeast at the 20 per cent level failed to gain as much weight as did the controls receiving 0.2 per cent yeast. The incidence of dental caries was significantly greater ($P = 0.04$) in the animals receiving 20 per cent yeast than in those receiving the control diet. Similarly, the severity of the lesions was greater in the animals receiving the high-yeast diet.

Histologic study of the major salivary glands and of the thyroid glands revealed no significant differences between the control and experimental groups in these structures. No microscopic evidence of hypothyroidism was found in the animals fed the high-yeast diet.

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